

pRNA CHIMERA

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This application claims the benefit of U.S. provisional patent application Ser. No. 60/433,697, filed Dec. 16, 2002, and is a continuation-in-part patent application of U.S. patent application Ser. No. 10/373,612, filed February 24, 2003, which claims priority to PCT/US01/26333, filed Aug. 23, 2001, which in turn claims the benefit of U.S. provisional patent application Ser. No. 60/227,393, filed Aug. 23, 2000, each of which patent applications is incorporated herein by reference in its entirety.

Statement of Government Rights

15 This invention was made with government support under grants from the National Institutes of Health (Grant No. GM59944 and Grant No. GM48159) and the National Science Foundation (Grant No. MCB-9723923). The government has certain rights in this invention.

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Background

A ribozyme is an RNA molecule capable of cleaving a target RNA molecule, or carrying out other catalytic and enzymatic functions. Structurally, it is single-stranded RNA characterized by two "arms" positioned either side of a small loop. The ribozyme base pairs to a region on the target RNA that is complementary to the nucleotide sequence of its two arms. The loop region serves as an active catalytic center that performs the cleaving function on the target RNA (Fig. 1).

The use of ribozymes for treatment and prevention of diseases in plants, humans and animals has the potential to revolutionize biotechnology. Hammerhead ribozymes have, for example, been used to cleave RNA in transgenic plants and animals. However, despite numerous publications reporting the results of investigations in test tubes, reports on the successful use of hammerhead ribozymes in living organisms are relatively few (Perriman et al., *Proc. Natl. Acad. Sci. USA* 92:6175-6179 (1995)). Although

it is clear that hammerhead ribozymes can cleave specific viral RNA or mRNA in test tubes, the efficiency of cleavage in cells is dramatically reduced due to instability and misfolding of the ribozyme in cells.

5 A major cause for the instability of ribozymes in an intracellular environment is degradation of the ribozyme by exonuclease present in the cells (Cotton et al., *EMBO J.* 8:3861-3866 (1989)). Exonucleases are enzymes that nonspecifically trim RNA from both ends. One method that has been used to block the intracellular degradation of ribozymes is to protect the ribozyme by connecting it at one end to a vector RNA, such as tRNA (Vaish et  
10 al., *Nucl. Acids Res.* 26:5237-5242 (1998)). However, due to refolding of the resulting chimera RNA, the ribozyme varied in efficiency compared to the unprotected ribozyme (Bertrand et al., *RNA* 3:75-88 (1997)). Tethering of a ribozyme to both ends of a tRNA has also been reported, but folding and/or activity was compromised (Vaish et al., *Nucl. Acids Res.* 26:5237-5242  
15 (1998)).

The potential to treat disease by using ribozymes to cleave RNA involved in cancer and pathogen infection is tremendous. The availability of a stabilized ribozyme that is resistant to degradation and is correctly folded such that it remains active in an intracellular environment would pave the way for  
20 the development of many important medical therapies.

#### Summary of the Invention

The invention provides a circularly permuted chimeric pRNA molecule carrying a stabilized, properly folded, biologically active moiety. The pRNA  
25 chimera is formed from a circularly permuted pRNA region, and a spacer region that includes the biologically active moiety. The biologically active moiety is not limited to any chemical structure but is preferably an RNA, such as a ribozyme, siRNA (small, interfering RNA), an RNA aptamer or an antisense RNA. The spacer region is covalently linked at its 5' and 3' ends to  
30 the pRNA region. Optionally, the spacer region includes first and second nucleotide strings interposed between the biologically active moiety and the pRNA region.

The invention circularly permuted chimeric pRNA of the invention can be monomeric or multimeric. When multimeric, the circularly permuted pRNA is preferably a dimer, a trimer or a hexamer, allowing the multimeric complex to be polyvalent. In a polyvalent multimeric complex, the multiple  
5 biologically active moieties may be the same or different. The multimeric complex may advantageously contain one or more biologically active moieties that facilitate specific targeting to deliver one or more therapeutic agents carried at other valency sites by the circularly permuted pRNA, such as biological moieties involved in cell surface binding, membrane diffusion or  
10 endocytosis. For example, the SELEX approach has been commonly used to screen for RNA aptamers that bind cell surface markers (Ellington et al., *Nature* 346, 818-822 (1990); Tuerk et al., *Science* 249, 505-510 (1990)). Such RNA aptamers can be attached to one of more subunits of the pRNA dimer, trimer or hexamer for specific cell recognition during delivery of the  
15 therapeutic agent. Other biologically active moieties that can be included in the multimeric complex include those involved in intracellular targeting and release of the therapeutic agent, and the like.

The pRNA region has a compact stable secondary structure characteristic of bacteriophage pRNA sequences. Thus, in one embodiment of  
20 the pRNA chimera, the pRNA region includes a circularly permuted pRNA of a bacteriophage selected from the group consisting of  $\phi$ 29, SF5', B103, PZA, M2, NF and GA1. In another embodiment of the pRNA chimera, the pRNA region includes:

- 25 (i) in the 5' to 3' direction beginning at the covalent linkage of the pRNA with the 3' end of the spacer region
  - a first loop;
  - a second loop; and
  - a lower stem-loop structure comprising a bulge, a first stem section and a third loop;
- 30 (ii) a second stem section interposed between the spacer region and the stem-loop structure;
- (iii) a third stem section interposed between the stem-loop structure and the first loop;

(iv) a fourth stem section interposed between the first loop and the second loop; and

(v) an opening defining 5' and 3' ends of the pRNA chimera, positioned anywhere within the pRNA region.

5       The invention also provides a method for making a pRNA chimera of the invention. A DNA encoding a pRNA chimera containing a pRNA region and a spacer region that includes a biologically active RNA is transcribed *in vitro* to yield the pRNA chimera. Optionally, the DNA encoding the pRNA chimera is generated using polymerase chain reaction on a DNA template, or  
10   the DNA is generated by cloning the DNA into a plasmid and replicating the plasmid.

      The invention further provides a method for determining whether an RNA molecule interacts with a test molecule. A pRNA chimera that includes the RNA molecule of interest is immobilized on a substrate, then contacted  
15   with test molecule. Whether or not the test molecule interacts with the RNA of interest, such as by binding the RNA of interest, is then detected.

      The invention also provides a DNA molecule that includes a nucleotide sequence that encodes a pRNA chimera containing a pRNA region and a spacer region that includes a biologically active RNA.

20       Also provided by the invention is a method for delivering a biologically active RNA to a cell, preferably a plant cell or an animal cell, such as human cell. In one embodiment, a DNA molecule having a nucleotide sequence that operably encodes a pRNA chimera of the invention is introduced into the cell and transcribed to yield the biologically active RNA.  
25   In another embodiment, the pRNA chimera is directly transfected into the cell. Alternatively, the chimeric RNA complex can be delivered to the cell via endocytosis by the incorporation of RNA aptamers that specifically bind to cell surface markers (Ellington et al., *Nature* 346, 818-822 (1990); Tuerk et al., *Science* 249, 505-510 (1990)).

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### Brief Description of the Figures

Figure 1 is a schematic depiction of target RNA cleavage by a representative ribozyme.

Figure 2 depicts the nucleotide sequence (SEQ ID NO:1) and  
 5 secondary structure of wild-type  $\phi$ 29 (phi29) pRNA indicating (a) the location and nomenclature of the loops and bulges (Zhang et al., *RNA* 3:315-323 (1997)); and (b) the procapsid binding domain and the DNA packaging domain; the right and left-hand loops, the head loop, the U<sup>72</sup>U<sup>73</sup>U<sup>74</sup> bulge, and the C<sup>18</sup>C<sup>19</sup>A<sup>20</sup> bulge are in boxes; the DNA-packaging domain (5'/3' ends) and  
 10 the procapsid binding domain (the larger area) are shaded; the curved line points to the two interacting loops; note that the three base UAA 3' overhang shown in (a) is absent in this diagram.

Figure 3 depicts that nucleotide sequences of several pRNAs prior to circular permutation: (a) bacteriophage SF5' (SEQ ID NO:11), (b)  
 15 bacteriophage B103 (SEQ ID NO:12), (c) bacteriophages  $\phi$ 29 and PZA (SEQ ID NO:1), (d) bacteriophage M2 and NF (SEQ ID NO:14), and (e) bacteriophage GA1 (SEQ ID NO:15) (Chen et al., *RNA* 5:805-818 (1999); and (f) aptRNA (SEQ ID NO:16).

Figure 4 is a schematic depiction of various structural features of a  
 20 pRNA chimera of the invention: (a) a whole pRNA chimera; (b) a spacer region component; (c) a pRNA region component.

Figure 5 is a schematic depiction of (a) the design of one embodiment of the pRNA chimera of the invention; and (b) exemplary circularly permuted pRNA (cpRNA) molecules showing various locations for the circle openings.

25 Figure 6 depicts (a) a possible mechanism of pRNA-ribozyme cleavage activity; and (b) the structural arrangement of the chimeric pRNA/ribozyme complex.

Figure 7 depicts (a) the sequence and predicted secondary structure of wild-type pRNA (SEQ ID NO:27); (b) the secondary structure of a pRNA  
 30 dimer (SEQ ID NO:26) (Trottier et al., *RNA* 6:1257-66 (2000)); (c) a three dimensional computer model of a pRNA dimer (Hoeprich and Guo, *J Biol Chem* 277:20794-803 (2002)); wherein the lines between residues of the monomer subunits of the dimer in (b) show the bases of the left and right hand

loops interact intermolecularly via hand-in-hand interaction (Guo et al., *Mol Cell* 2:149-55 (1998); Zhang et al., *Mol Cell* 2:141-47 (1998)); (d) and (e) diagrams depicting the formation of a pRNA hexameric ring by upper and lower loop sequence interaction.

5           Figure 8 depicts various embodiments of a pRNA dimer, trimer and hexamer as a polyvalent gene delivery vector.

Figure 9 presents the impact of various extensions (SEQ ID NOs:19-22) of the 3' end of the pRNA on viral activity as measured by plaque forming units.

10           Figure 10 depicts the design and production of circularly permuted pRNAs. The DNA template in (a) (SEQ ID NO:2) uses a short (AAA) sequence to join the native 5'/3' ends, while the template in (b) (SEQ ID NO:13) uses a longer sequence (SEQ ID NO:8) to join the native 5'/3' ends. New openings of the cpRNA are indicated by the wedges pointing to places in  
15 the transcript sequence. (See Zhang et al., *RNA* 3:315-323 (1997)).

Figure 11 depicts an RNA chimera (SEQ ID NO:3) bound to a portion of the U7snRNA substrate (SEQ ID NO:4).

Figure 12 depicts *in vitro* cleavage of substrates by chimeric ribozyme carried by pRNA. (a) Schematic showing secondary structure of RzU7, the  
20 U7 snRNA targeting ribozyme (SEQ ID NO:10), base-pairing with its substrate (SEQ ID NO:4). (b) Denaturing urea gel showing cleavage of the substrate U7snRNA into its expected 69mer and 25mer cleavage products by both the ribozyme RzU7 and the chimera ribozyme PRNA-RzU7.

Figure 13 depicts a denaturing urea gel evidencing successful cleavage  
25 of the substrate HBV-polyA into its expected 70mer and 67mer cleavage products.

Figure 14 depicts the design and construction of plasmid encoding the self-process ribozyme targeting at the HBV polyA signal. (a) shows the design of plasmid encoding ribozyme pRNA-RzA. (b) shows the processed  
30 chimeric ribozyme after transcription and cis-cleavage. (c) shows the secondary structure of the hammerhead ribozyme (RzA) (SEQ ID NO:23) base paired to the HBV polyA target sequence (SEQ ID NO:24). An indicated change from "G" to "A" generated an inactive enzyme as negative control. (d)

shows secondary structure of the ribozyme pRNA-RzA (SEQ ID NO:25) base paired to the HBV polyA substrate (SEQ ID NO:24).

Figure 15 depicts the effect of ribozymes on HBV RNA levels in HepG2 cells.

5        Figure 16 depicts an anti-12-LOX ribozyme (SEQ ID NO:5) bound to substrate RNA (SEQ ID NO:6).

Figure 17 depicts the use of circularly permuted pRNA in the SELEX method to identify RNA aptamers that bind to a pre-identified substrate.

NNN. . . N(25-100) . . . NNN, random sequence of template; template,  
10    template primer; primer 1, 3' end primer; primer 2, 5' end primer.

Figure 18 depicts formation of pRNA dimer, trimer and hexamer via the interaction of the right (uppercase letter) and left (lower case letter) hand loop. The same letters in upper and lower cases, e.g. A and a', indicate complementary sequences, while different letters, e. g. A and b', indicate non-  
15    complementary loops.

Figure 19 depicts secondary structure, domain and location of pRNA on phi29 viral particle: (a) secondary structure of pRNA A-b'. The intermolecular binding domain (shaded area) and the reactive DNA translocation domain are marked with bold lines. The four bases in the right  
20    and left loops, which are responsible for inter-RNA interactions, are boxed; (b) Power Rangers depict pRNA hexamer by hand-in-hand interaction; (c) phi29 DNA-packaging motor with pRNA hexamer formed by pRNA A-b' and B-a'. The surrounding pentagon stands for the fivefold symmetrical capsid vertex, viewed as end-on with the virion at side-view. The central region of  
25    pRNA binds to the connector and the 5'/3' paired region extends outward (Chen et al., *RNA*, 5:805-818 (1999)).

Figure 20 depicts (a) two; (b) three and (c) six interlocking pRNAs.

Figure 21 depicts restoration of cell-cell contacts of breast cancer cells (MDA-231) infected with adenovirus expressing EphrinA1-Fc, as detected by  
30    fluorescence using an anti-EphA2 antibody. MCF-10A, normal human breast epithelial cells. The normal cell would exhibit cell-cell contact and form a monolayer; the cancer cell would form a colony.

Figure 22 depicts a representative chimeric pRNA design. I. The chimeric pRNA harboring a ribozyme hybridized to a target. II. The secondary structure of a pRNA monomer. III. The secondary structure of a chimeric pRNA harboring a ribozyme targeting an HIV tat/rev substrate. IV. The secondary structure of a chimeric pRNA harboring an adenovirus knob-binding aptamer. V. The secondary structure of a chimeric pRNA harboring a CD4-binding RNA aptamer. The three pRNAs combine to form a trimer.

Figure 23 shows silencing of gene for green fluorescence protein (GFP) using a pRNA/siRNA chimeric complex targeting GFP (a) fluorescence images showing left, chimeric complex; middle, siRNA alone; right; control; (b) Northern analysis, lanes 1, 2, 9 and 10, chimeric complex; lanes 3, 7, siRNA alone; lanes 4, 8, control.

Figure 24 shows the viability of cancer cells in the absence (left) and presence (right, showing cell death) of a pRNA/ribozyme chimeric RNA complex that targets survivin.

#### Detailed Description

Bacteriophage  $\phi 29$  (phi29) is a double-stranded DNA virus. In 1987, one of the inventors, Dr. Peixuan Guo, discovered a viral-encoded 120 base RNA that plays a key role in bacteriophage  $\phi 29$  DNA packaging (Guo et al. *Science* 236:690-694 (1987)). This RNA is termed packaging RNA or "pRNA". It binds to viral procapsids at the portal vertex (the site where DNA enters the procapsid) (Guo et al., *Nucl. Acids Res.* 15:7081-7090 (1987)) and is not present in the mature  $\phi 29$  virion.

Six copies of pRNA are needed to package one genomic DNA (Trottier et al., *J. Virol.* 70:55-61 (1996); Trottier et al., *J. Virol.* 71, 487-494 (1997); Guo et al., *Mol. Cell.* 2, 149-155 (1998)). DNA packaging is completely blocked when one of the six slots is occupied by one inactive pRNA with a mutation at the 5' or 3' end (Trottier et al., *J. Virol.* 70:55-61 (1996); Trottier et al., *J. Virol.* 71:487-494 (1997)). Bacteriophage  $\phi 29$  pRNA is associated with procapsids during the DNA translocation process (Chen et al., *J. Virol.* 71:3864-3871 (1997)). Inhibition data also suggests that the pRNA plays an essential role in DNA translocation (Trottier et al., *J. Virol.*



71:487-494 (1997)); Trottier et al. *J. Virol.* 70:55-6 (1996)). A  $Mg^{2+}$ -induced conformational change of pRNA leads to its binding to the portal vertex (Chen et al. *J. Virol.* 71, 495-500 (1997)). The tertiary structure of the pRNA monomer and dimer has also reported (Zhang et al., *Virology* 81:281-93 (2001); Trottier et al., *RNA* 6(9):1257-1266 (2000); Chen et al. *J. Biol. Chem.* 275(23): 17510-17516 (2000); Garver et al., *J. Biol. Chem.* 275(4): 2817-2824 (2000)).

Recently, a computer model of the three-dimensional structure of a pRNA monomer has been constructed (Hoeprich and Guo, *J. Biol. Chem.* 277:20794-803 (2002)) based on experimental data derived from photo-affinity cross-linking (Garver and Guo, *RNA* 3:1068-79 (1997); Chen and Guo, *J Virol* 71:495-500(1997)); chemical modification and chemical modification interference (Mat-Arip et al., *J Biol Chem* 276:32575-84 (2001); Zhang et al., *Virology* 281:281-93 (2001); Trottier et al., *RNA* 6:1257-66 (2000)); complementary modification (Zhang et al., *RNA* 1:1041-50 (1995); Zhang et al., *Virology* 201:77-85 (1994); Zhang et al., *RNA* 3:315-22 (1997); Reid et al., *J Biol Chem* 269:18656-61 (1994); Wichitwechkarn et al., *Mol Biol* 223:991-98 (1992)); nuclease probing (Chen and Guo, *J Virol* 71:495-500 (1997); Reid et al., *J Biol Chem* 269:5157-62 (1994); Zhang et al., *Virology* 211:568-76 (1995)); oligo targeting competition assays (Trottier and Guo, *J Virol* 71:487-94 (1997); Trottier et al., *J Virol* 70:55-61(1996)) and cryo-atomic force microscopy (Mat-Arip et al., *J Biol Chem* 276:32575-84 (2001); Trottier et al., *RNA* 6:1257-66 (2000); Chen et al., *J Biol Chem* 275:17510-16 (2000)). pRNA hexamer docking with the connector crystal structure reveals a very impressive match with available biochemical, genetic, and physical data concerning the 3D structure of pRNA (Hoeprich and Guo, *J Biol Chem* 277:20794-803 (2002)).

The nucleotide sequence (SEQ ID NO:1) of native full length  $\phi$ 29 pRNA (Guo et al., *Nucl. Acids Res.* 15:7081-7090 (1987)), as well as its predicted base-paired secondary structure, is shown in Fig. 2(a) (Zhang et al., *RNA* 3:315-323 (1997); Zhang et al., *Virology* 207:442-451 (1995)). The predicted secondary structure has been partially confirmed (Zhang et al., *RNA* 1:1041-1050 (1995); Reid et al., *J. Biol. Chem.* 269:18656-18661 (1994);

Zhang et al., *Virology* 201:77-85 (1994); Chen et al., *J. Virol.* 71: 495-500 (1997)).

As shown in Fig. 2(b), the pRNA monomer contains two functional domains, the procapsid binding domain and the DNA translocating domain.

5 The procapsid binding domain is located at the central part of the pRNA molecule at bases 23-97 (Garver et al., *RNA* 3:1068-79 (1997); Chen et al., *J Biol Chem* 275:17510-16 (2000)), while the DNA translocation domain is located at the 5'/3' paired ends. The 5' and 3' ends have been found to be proximate, and several kinds of circularly permuted pRNA have been

10 constructed (Zhang et al., *RNA* 3:315-22 (1997); Zhang et al., *Virology* 207:442-51 (1995); Guo, *Prog in Nucl Acid Res & Mole Biol* 72:415-72 (2002)). These two domains are compact and fold independently, suggesting that exogenous RNA can be connected to the end of the pRNA without affecting pRNA folding and that phi29 pRNA could be used as a vector to

15 escort and chaperone small therapeutic RNA molecules. Indeed, removal of the DNA translocating domain does not change the nature of pRNA's intermolecular interaction, i.e., replacement or insertion of nucleotides before residue #23 or after residue #97 does not interfere with the formation of dimers, trimers, and hexamers (Hoeprich et al., *Gene Therapy*, 10(15):1258-

20 1267 (2003); Chen et al., *RNA*, 5:805-818 (1999); and Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003)). We have confirmed that exogenous RNA can be connected to the 3' or 5' end of the pRNA without affecting pRNA folding; this foreign RNA molecule also folds independently (Hoeprich et al., *Gene Therapy*, 10(15):1258-1267 (2003) and Shu et al., *J Nanosci and*

25 *Nanotech (JNN)*, 4:295-302 (2003)).

Phylogenetic analysis of pRNAs from phages SF5', B103,  $\phi$ 29, PZA, M2, NF and GA1 (Chen et al., *RNA* 5:805-818 (1999)) shows very low sequence identity and few conserved bases, yet the family of pRNAs appear to have strikingly similar and stable predicted secondary structures (Fig. 3). The

30 pRNAs from bacteriophages SF5' (SEQ ID NO:11), B103 (SEQ ID NO:12),  $\phi$ 29/PZA (SEQ ID NO:1), M2/NF (SEQ ID NO:14), GA1 (SEQ ID NO:15) of *Bacillus subtilis* (Chen et al., *RNA* 5:805-818 (1999); and aptRNA (SEQ ID NO:16) are all predicted to have a secondary structure that exhibits essentially

the same structural features as shown in Fig. 2 for  $\phi$ 29 pRNA (Chen et al., *RNA* 5:805-818 (1999)). All have native 5' and 3' ends at the left end of a stem structure (as shown in Fig. 3) and contain the same structural features positioned at the same relative locations.

5           The pRNA of these bacteriophages, sharing as they do a single stable secondary structure, provide the framework for the pRNA chimera of the invention.

          Secondary structure in an RNA molecule is formed by base pairing among ribonucleotides. RNA base pairs commonly include G-C, A-T and U-  
10   G. Predictions of secondary structure are preferably made according to the method of Zuker and Jaeger, for example by using a program known by the trade designation RNASTRUCTURE 3.6, written by David H. Mathews (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999); see also Zuker, *Science* 244:48-52 (1989); Jaeger et al., *Proc. Natl. Acad. Sci. USA* 86:7706-7710  
15 (1989); Jaeger et al., *Meth. Enzymol.* 183:281-306 (1990)). This program is publicly available on the worldwide web at the homepage of the laboratory of Douglas Turner at the University of Rochester at [rna.chem.rochester.edu/RNAstructure.html](http://rna.chem.rochester.edu/RNAstructure.html) and runs on MS Windows 95, 98, ME, 2000 and NT4. The program is also publicly available on the worldwide  
20 web at Michael Zuker's homepage at Rensselaer Polytechnic Institute ([bioinfo.math.rpi.edu/~zukerm/home.html](http://bioinfo.math.rpi.edu/~zukerm/home.html)); his homepage offers online folding and a version of the algorithm that can be compiled on Silicon Graphics, Sun, or DEC Alpha workstations. The structure with the lowest energy (i.e., the optimal structure) is chosen.

25           Secondary structures of RNA can be characterized by stems, loops and bulges. A "stem" is a double-stranded section of two lengths of base-paired ribonucleotides. Stem sections contain at least 2 base pairs and are limited in size only by the length of the RNA molecule. A "loop" is a single-stranded section that typically includes at least 3 ribonucleotides and is also limited in  
30 size only by the length of the RNA molecule. In a "stem loop", the 5' and 3' ends of the loop coincide with the end of a base-paired stem section. In a "bulge loop", the loop emerges from along the length of a stem section. The 5' and 3' ends of a bulge loop are typically not base paired although they may

potentially be (see, e.g., G40 and C48 of the bulge loop in the  $\phi$ 29 pRNA structure; Fig. 2). A "bulge" is an unpaired single stranded section of about 1 to about 6 ribonucleotides present along the length of (or between) stem sections. Note that there is no clear line between a large "bulge" and a small "bulge loop." Herein, where the term "bulge" is used, it also includes a small "bulge loop" (i.e., a bulge loop of less than about 7 ribonucleotides).

The secondary structure of an RNA molecule is determined by the nature and location of the base pairing options along its length. RNA secondary structure is degenerate; that is, different primary ribonucleotide sequences can yield the same base pairing configurations and hence the same secondary structure. In a way, it is akin to the way multiple amino acid sequences can produce the same secondary structure, for example an  $\alpha$ -helix.

A single secondary structure is dictated by a number of different primary sequences in predictable and well-understood ways. For example, single or pairs of nucleotides can generally be added, removed, or substituted without altering the overall base pairing interactions within the RNA molecule and without interfering with its biological function. This is particularly true if one or a few base pairs of nucleotides are removed, added or substituted along double-stranded hybridized length of the molecule, or if one or more nucleotides is removed, added or substituted in the single-stranded loop regions. For example, although GC base pairs and AT base pairs differ slightly in their thermodynamic stability, one can generally be substituted for another at a site within the double-stranded length without altering the secondary structure of an RNA molecule. GC base pairs are preferred in the stem region due to their added stability. Changes in secondary structure as a result of addition, deletion or modification of nucleotides can be readily assessed by applying the secondary structure prediction algorithm of Zuker and Jaeger as described above. The pRNA region of the RNA chimera can accommodate substantial variation in primary sequence without an appreciable change in secondary structure.

The pRNA chimera of the invention consists essentially of a pRNA region having the secondary structure exemplified in Fig. 3 (and schematically depicted in Fig. 4, as detailed below), interrupted by (i.e., flanking) a

heterologous spacer region that contains a biologically active moiety, such as a ribozyme. The secondary structure of the pRNA region of the pRNA chimera is the common secondary structure that characterizes the pRNA from bacteriophages  $\phi$ 29, SF5', B103, PZA, M2, NF and GA1. The spacer region  
5 is termed "heterologous" because all or a portion of its nucleotide sequence is engineered or it is obtained from an organism other than the bacteriophage. It is the presence of the heterologous spacer region that renders the construct "chimeric" for the purposes of this invention. The pRNA chimera is useful as a vehicle to carry and deliver a ribozyme or other biologically active moiety to  
10 a target molecule or location. Since both ends of the ribozyme are connected to pRNA, the linkage is expected to protect the sensitive ribozyme from degradation and to assist the biologically active moiety to fold appropriately.

Notably, the ability of the pRNA chimera to perform its intended function of protecting and carrying a biologically active moiety depends not  
15 on the primary nucleotide sequence of the pRNA region (the primary structure), but on the secondary structure (base pairing interactions) that the pRNA region assumes as a result of its primary ribonucleotide sequence. The "pRNA region" of the pRNA chimera is so termed because it has a secondary structure, although not necessarily an RNA sequence, characteristic of a native  
20 bacteriophage pRNA molecule. Therefore, unless otherwise specified, the term "pRNA region" as used herein includes naturally occurring (native) pRNA sequences, nonnaturally occurring (nonnative) sequences, and combinations thereof provided that they yield the secondary structure characteristic of naturally occurring (native) bacteriophage pRNA as described  
25 herein. Stated another way, the term "pRNA region" is not intended to be limited to only those particular nucleotide sequences native to pRNA. The pRNA region can thus contain any nucleotide sequence which results in the secondary structure shown in Fig. 4. Nucleotide sequences that fold into the aforesaid secondary structure include naturally occurring sequences, those that  
30 are derived by modifying naturally occurring pRNA sequences, and those that are designed *de novo*, as well as combinations thereof. One of skill in the art can readily determine whether a nucleotide sequence will fold into the secondary structure shown in Fig. 4 and described herein by applying a

secondary structure algorithm, such as RNASTRUCTURE as described above, to the nucleotide sequence.

Examples of nucleotide sequences that, when folded, yield the secondary structure of the pRNA region of the pRNA chimera of the invention are shown in Fig. 3. They include pRNA sequences from bacteriophages SF5' (SEQ ID NO:11), B103 (SEQ ID NO:12),  $\phi$ 29/PZA (SEQ ID NO:1), M2/NF (SEQ ID NO:14), GA1 (SEQ ID NO:15) as well as the aptRNA (SEQ ID NO:16).

In embodiments of the pRNA chimera wherein the pRNA region includes or is derived from a naturally occurring pRNA, the spacer region of the pRNA chimera is covalently linked to the pRNA region at what can be considered the "native" 5' and 3' ends of a pRNA sequence, thereby joining the native ends of the pRNA region. The pRNA region of the pRNA chimera is optionally truncated when compared to the native bacteriophage pRNA; in those embodiments, and that as a result the "native" 5' and 3' ends of the pRNA region simply refer to the nucleotides that terminate or comprise the actual end of the truncated native pRNA. An opening is formed in the pRNA region to linearize the resulting pRNA chimera, effecting a "circular permutation" of the pRNA as detailed below. It should nonetheless be understood that the term "circularly permuted pRNA region" is not limited to naturally occurring pRNAs that have been circularly permuted but instead is intended to have the broader meaning of RNA having a pRNA-like secondary structure as shown in Fig. 4(c), including an opening in the pRNA region that forms the 5' and 3' ends of the pRNA chimera.

Examples of pRNA chimera of the invention are those formed from the pRNAs of bacteriophages SF5' (SEQ ID NO:11), B103 (SEQ ID NO:12),  $\phi$ 29/PZA (SEQ ID NO:1), M2/NF (SEQ ID NO:14), GA1 (SEQ ID NO:15) as well as aptRNA (SEQ ID NO:16) by joining the native 5' and 3' ends to the spacer region and introducing an opening elsewhere in the pRNA region, as described herein. Another example of a pRNA chimera of the invention is:

5'-GUUGAUN<sub>j</sub>GUCAAUCAUGGCAA-spacer region-  
UUGUCAUGUGUAUGUUGGGGAUUAN<sub>j</sub>CUGAUUGAGUUCAGCCCAC  
AUAC-3' (SEQ ID NO:7)

where N represents any nucleotide, without limitation and j is an integer between about 4 to about 8. Preferably j is 4 or 5. The spacer region is represented by  $N_m - B - N_n$  where  $N_n$  and  $N_m$  are nucleotide strings that are optionally included in the spacer region, and B includes the biologically active moiety. Preferably, B is a ribonucleotide sequence that includes a biologically active RNA. Both m and n can be independently zero or any integer. Preferably, m and n are independently at least about 3, more preferably at least about 5, and most preferably at least about 10. Further, n and m are independently preferably at most about 300, more preferably at most about 50, and most preferably at most about 30.

Further, since the pRNA region of the pRNA chimera is defined by its secondary structure, still other examples of a pRNA chimera can be readily made by "mixing and matching" nucleotide fragments from, for example, SEQ ID NO:s 1, 2, 7, 11, 12, 14, 15 and 16 that fold into particular secondary structural features (bulges, loops, stem-loops, etc.) provided that the resulting nucleotide sequence folds into the overall secondary structure as shown in Fig. 4. For example, nucleotides encoding bulge loop 22 from bacteriophage SF5' pRNA (SEQ ID NO:11) could be substituted for the nucleotides encoding bulge loop 22 in the  $\phi$ 29 pRNA (SEQ ID NO:1) to yield a pRNA region as described herein. Likewise, any number of artificial sequences can be substituted into SEQ ID NO:s 1, 2, 7, 11, 12, 14, 15 and 16 to replace nucleotide sequences that fold into one or more structural features (or portions thereof) to form a pRNA region as described herein. See, for example, aptRNA (Fig. 3(f)) which was derived in that fashion from  $\phi$ 29 pRNA. The overarching principle is that the overall secondary structure of the pRNA region is the secondary structure common to the bacteriophage pRNAs, as schematically depicted in Fig. 4.

Importantly, the resulting pRNA chimera is not a circular molecule; rather, it is linearized due to a circular permutation of the pRNA region (Zhang et al., *RNA* 3:315-323 (1997); Zhang et al., *Virology* 207:442-451 (1995)). Briefly, an opening (i.e., a cleavage or break point) is provided in the pRNA region at any designated site to form the actual 5' and 3' ends of the

RNA chimera. These 5' and 3' ends are at "nonnative" positions with respect to a naturally occurring linear pRNA.

Fig. 5(a) shows how a pRNA chimera of the invention can be formed from a ribozyme and a pRNA region. The 5' and 3' ends of the pRNA can be engineered into any desired site on the circularly permuted pRNA chimera. Fig. 5(b) shows exemplary circularly permuted RNA molecules showing various locations for the circle openings.

Fig. 4 depicts various structural features that characterize a pRNA chimera of the invention. As shown in Fig. 4(a), the linear molecule includes a pRNA region 1 and a spacer region 2. Spacer region 2 contains a biologically active moiety 3, in this case a ribozyme, flanked by ribonucleotide strings 4. The pRNA region 1 is bifurcated; it includes a first pRNA segment 5 having 3' end 6 and "native" 5' end 7, and a second pRNA segment 8 having "native" 3' end 9 and 5' end 10. Ends 6 and 10 are the actual terminal ends of the pRNA chimera. Opening 11 renders the molecule linear and can be positioned anywhere in pRNA region 1 by the relocation of ends 6 and 10.

Spacer region 2 is shown in detail in Fig. 4(b). Ribozyme 3 is composed of a catalytic domain 15 flanked by target-binding sequences 16. pRNA region 1 is shown in detail in Fig. 4(c). Overall, pRNA region 1 is characterized by a stem-loop secondary structure, wherein loop 24 is relatively small and the base-pairing in the stem (essentially stem sections 20, 21 and 23) is interrupted by structures on either side of loop 24. Bulge loop 22 is positioned 5' of loop 24. Positioned 3' of loop 24 is a stem-loop structure that contains bulge 25, stem 26 and loop 27.

Stem section 20 can be any number of ribonucleotides in length and can contain an unlimited number of bulges provided it is still able to base pair. Preferably, stem section 20 contains at least about 4, more preferably at least about 10 base pairs; further, it preferably it contains at most about 50, more preferably at most about 40 base pairs. Preferably stem section 20 contains about 0 to about 8 bulges; more preferably it contains about 0 to about 4 bulges.



Stem section 21 preferably contains 5-13 base pairs and 0-2 bulges.

Bulge loop 22 preferably contains 5-12 bases.

Stem section 23 preferably contains 3-12 base pairs and 0-2 bulges.

Loop 24 preferably contains 3-8 bases.

5 Bulge 25 preferably contains 0-5 bases.

Stem 26 preferably contains 4-8 base pairs and 0-2 bulges.

Loop 27 preferably contains 3-10 bases.

Tertiary interactions within an RNA molecule may result from nonlocal interactions of areas of the RNA molecule that are not near to each other in the primary sequence. Although native bacteriophage pRNA appears to exhibit tertiary interactions between bulge loop 22 and loop 27 (Chen et al., *RNA* 5:805-818 (1999); Guo et al, *Mol. Cell.* 2:149-155 (1998)) it should be understood that the pRNA chimera of the invention is not limited to RNA molecules exhibiting any particular tertiary interactions.

15 In one embodiment, the pRNA chimera of the invention contains at least 8, more preferably at least 15, most preferably at least 30 consecutive ribonucleotides found in native SF5' pRNA (Fig. 3(a)), B103 pRNA (Fig. 3(b)),  $\phi$ 29/ PZA pRNA (Fig. 3(c)), M2/NF pRNA (Fig. 3(d)), GA1 pRNA (Fig. 3(e)), or aptRNA (Fig. 3(f)), preferably native  $\phi$ 29 pRNA. Most preferably, the pRNA region of the pRNA chimera contains at least a  $\phi$ 29 pRNA sequence that starts at ribonucleotide 23, preferably at ribonucleotide 20, and ends at ribonucleotide 95, preferably ribonucleotide 97, in the  $\phi$ 29 pRNA sequence (Fig. 2). In addition or in the alternative, the nucleotide sequence of the pRNA region of the pRNA chimera is preferably at least 60% identical to, more preferably 80% identical to, even more preferably 90% identical to, and most preferably 95% identical to the nucleotide sequence of a corresponding native SF5' pRNA (Fig. 3(a)), B103 pRNA (Fig. 3(b)),  $\phi$ 29/ PZA pRNA (Fig. 3(c)), M2/NF pRNA (Fig. 3(d)), GA1 pRNA (Fig. 3(e)), or the aptRNA chimera (Fig. 3(f)), most preferably  $\phi$ 29 pRNA (particularly bases 20-97).

Percent identity is determined by aligning two polynucleotides to optimize the number of identical nucleotides along the lengths of their sequences; gaps in either or both sequences are permitted in making the

alignment in order to optimize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their proper order. For example, the two nucleotide sequences are readily compared using the Blastn program of the BLAST 2 search algorithm, as described by Tatusova et al. (*FEMS Microbiol Lett* 1999, 174:247-250). Preferably, the default values for all BLAST 2 search parameters are used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x\_dropoff = 50, expect = 10, wordsize = 11, and filter on.

The covalent linkages between the biologically active moiety and the pRNA region can be direct or indirect but preferably are indirect. In an indirect linkage, the spacer region includes additional string(s) of ribonucleotides at one or both ends of the biologically active moiety. These ribonucleotide strings, if present, contain preferably at least about 3 ribonucleotides; and preferably contain at most about 300, more preferably at most about 30 ribonucleotides. Compositionally, the strings can contain any desired ribonucleotides, however it is preferably that ribonucleotide compositions are selected so as to prevent the ribonucleotide strings on either side of the biological moiety from base pairing with each other or with other parts of the pRNA chimera.

Exemplary biologically active moieties include, without limitation, DNA, RNA, DNA or RNA analogs, including a ribozyme, a siRNA, an RNA aptamer, or an antisense RNA, peptide nucleic acid (PNA), a peptide, a protein such as an antibody, a polysaccharide, a lipid, a virus, a plasmid, a cofactor, or a combination thereof. Since siRNA is a double-stranded RNA, the effective siRNA moiety could include any sequence to replace the 5'/3' paired helical region. Preferably the biological activity of the biologically active moieties is an enzymatic activity or binding activity or both; for example, the biologically active moiety may function as or encode a ribozyme or other catalytic moiety.

The biologically active moiety is preferably a polynucleotide. A preferred biologically active polynucleotide is a polyribonucleotide, more preferably the biologically active polynucleotide is a ribozyme such as a hammerhead ribozyme or a hairpin ribozyme. Antisense RNA and other bioactive RNAs are also preferred.

It should be understood that the terms "nucleotide," "oligonucleotide," and "polynucleotide" as used herein encompass DNA, RNA, or combinations thereof, unless otherwise indicated. Further, the terms DNA and RNA should be understood to include not only naturally occurring nucleic acids, but also sequences containing nucleotide analogs or modified nucleotides, such as those that have been chemically or enzymatically modified, for example DNA phosphorothioates, RNA phosphorothioates, and 2'-O-methyl ribonucleotides. Nucleotide derivatives, such as 2'-F-2' deoxy CTP and 2'-F-2' deoxy UTP, are optionally incorporated into the RNA to produce stable *in vitro* RNA transcripts that are resistant to RNase digestion. Since biological function of the pRNA itself (other than its ability to form multimeric complexes) is not a concern, inclusion of non-natural nucleotide derivatives is suitable, especially for the receptor-binding aptamers selected from a random pool (e.g., using SELEX). If the incorporation of RNase-resistant nucleotide derivatives into a therapeutic RNA tethered to the pRNA does happen to interfere with the catalytic function of the cargo RNA, the cargo RNA can be synthesized with regular nucleotides and ligated to the pRNA molecule.

A ribozyme is generally characterized by:

arm 1 - active enzyme center - arm 2

where arm 1 and arm 2 are sequences complementary to the target substrate to be cleaved by the ribozyme, and the active enzyme center is the catalytic center that cleaves the target RNA. The "arms" of the ribozyme typically contain at least about 7 nucleotides, preferably at least about 12 nucleotides; and typically contain at most about 100 nucleotides, preferably at most about 30 nucleotides. The nucleotide sequence of the arms can be engineered to hybridize to the nucleotide sequence of any desired target nucleic acid.

Advantageously, incorporating a biologically active polynucleotide, e.g., a ribozyme, into the pRNA chimera of the invention protects the ends of the ribozyme thereby rendering it resistant to exonuclease degradation. Moreover, the secondary structure of pRNA is compact and very stable. A

pRNA domain defined by nucleotides 30-91 of  $\phi$ 29 pRNA is especially stable.

The compactness and stability of pRNA allows the pRNA region and the ribozyme to fold independently. Proper folding of the inserted RNA is facilitated, thereby preserving its biological activity. The stable structure of the carrier pRNA region is retained as well. A major obstacle in designing molecules to deliver ribozymes, i.e., misfolding of the ribozyme and carrier region as a result of interactions between them, has thus been overcome by utilizing the very stable pRNA molecule as the carrier. That the activity of the ribozyme is retained in the circularly permuted pRNA chimera is especially significant because it means that the new 5' and 3' ends of the pRNA chimera can be positioned so as to "bury" them in the folded pRNA structure, thereby further protecting the pRNA chimera from degradation. These features suggest great promise for the use of the pRNA chimera of the invention as a ribozyme delivery vehicle in medical and veterinary applications.

As shown in the Examples below, circularly permuted pRNAs were constructed without impacting pRNA folding. In addition, connecting the pRNA 5'/3' ends with variable sequences did not disturb its folding and function. These unique features, which help prevent two common problems - exonuclease degradation and misfolding in the cell, make pRNA an ideal vector to carry therapeutic RNAs.

The pRNA chimera of the invention employs a "circular permutation" of a bacteriophage pRNA. A "circularly permuted" RNA molecule (cpRNA) is a linear RNA molecule in which the native 5' and 3' ends are covalently linked. The linkage can be direct, or it can be indirect by using a spacer region. Since a cpRNA molecule is linear, new nonnative 5' and 3' ends are created by forming an opening in the molecule (i.e., a discontinuity in the pRNA sequence) at a different location. The pRNA chimera of the invention is linear as a result of a nonnative opening in the bacteriophage pRNA framework at a designated site, which circularly permutes the bacteriophage framework and forms the actual 5' and 3' ends of the pRNA chimera. As already noted, the nonnative opening can be at any desired location in the pRNA region. Examples of selected locations in, for example in  $\phi$ 29 pRNA

can be found in Zhang et al., *RNA* 3:315-323 (1997) and Zhang et al., *Virology* 207:442-451 (1995). See also Garver et al., *J. Biol. Chem.* 275:2817-2824 (2000); Chen et al., *J. Virology* 71:495-500 (1997); Trottier et al., *RNA* 6:1257-1266 (2000); and Zhang et al., *Virology* 281:281-293 (2001).

5           The pRNA chimera of the invention can be synthesized chemically or enzymatically using standard laboratory protocols. The pRNA region is preferably transcribed from a DNA template that encodes it, although if desired it can be synthesized chemically. If synthesized chemically, the pRNA region optionally contains nonnative nucleotides (e.g., derivatized or  
10 substituted nucleotides) and/or nonnative bonds analogous to the phosphodiester bonds that characterize naturally occurring nucleic acids.

          Preferably the pRNA region is transcribed or synthesized as a single RNA molecule. In one embodiment of the method, the spacer region is chemically or enzymatically linked to the "native" ends of the pRNA region to  
15 form a circular chimeric molecule. The pRNA is then cleaved at a predetermined site to form the linear, circularly permuted pRNA chimera.

          When the spacer region is RNA, another embodiment of the method includes transcribing the entire pRNA chimera from a single DNA template that encodes the entire chimeric molecule. In another embodiment of the  
20 method, the RNA spacer region is produced separately, either via transcription from its own template or by chemical synthesis, after which it is ligated to the pRNA region.

          Also included in the invention is a DNA molecule that includes a nucleotide sequence that encodes the pRNA chimera of the invention. The  
25 spacer region of the encoded chimera is necessarily RNA in this aspect of the invention. The DNA molecule can be linear or circular. It can be double stranded or single stranded; if single stranded, its complement is included in the term "DNA molecule" as well.

          The pRNA chimera of the invention can be introduced into a host cell  
30 in a number of different ways. For example, the pRNA chimera can be synthesized outside the cell, contacted with the cell surface such that a constituent RNA aptamer or other targeting agent binds to a component of the cell surface, and taken up by the cell via receptor-mediated endocytosis,

membrane diffusion, transport through a pore, or the like. Alternatively, it can be delivered as part of the genetic cargo carried by a viral delivery agent (either an RNA virus or a DNA virus). It can also be delivered as a plasmid, i.e., as a DNA molecule that encodes the desired pRNA chimera. It is also possible to directly transfect the pRNA chimera into the host cell. For example, a product available under the trade designation TRANSMESSENGER TRANSFECTION REAGENT (available from Qiagen), which is a lipid-based formulation that is used in conjunction with a specific RNA-condensing enhancer and an optimized buffer, can be used to transfect the pRNA chimera into eukaryotic cells.

A DNA molecule for use in introducing a pRNA into a cell preferably contains regulatory elements such that the pRNA chimera is operably encoded. A pRNA chimera is "operably encoded" by a DNA molecule when the DNA molecule contains regulatory elements that allow the pRNA chimera to be produced by transcription of the DNA molecule inside the cell. Such regulatory elements include at least a promoter. Optionally, the DNA molecule includes additional regulatory motifs that promote transcription of the RNA chimera, such as, but not limited to, an enhancer. The DNA molecule can be introduced into the host cell using anionic or cationic lipid-mediated delivery or other standard transfection mechanisms including electroporation, adsorption, particle bombardment or microinjection, or through the use of a viral or retroviral vector.

Optionally, the DNA molecule can contain one or more features that allow it to integrate into the cell's genome. For example, it can be delivered in the form of a transposon, a retrotransposon, or an integrating vector; alternatively, it can contain sequences that are homologous to genomic sequences that allow it to integrate via homologous recombination. On the other hand, the DNA molecule can be designed to exist within a cell as nongenomic DNA, e.g., as a plasmid, cosmid, episome and the like.

Transcription from a DNA template encoding the entire chimeric RNA molecule can occur *in vitro* or within a cell. The cell can be in cell culture, or in an organism (*in vivo*) such as a plant or an animal, especially a human, or in a cell explanted from an organism (*ex vivo*).

Advantageously, the pRNA chimera of the invention can be used to deliver a biologically active RNA molecule to a target within a cell. A DNA molecule having nucleotide sequence that operably encodes a circularly permuted pRNA region and a spacer region is introduced into a cell. The spacer region includes a biologically active RNA, and transcription of the DNA to yields the biologically active RNA. The biologically active molecule thus delivered is preferably a ribozyme, and the target is preferably viral or mRNA associated with a gene whose expression it is desirable to reduce. Fig. 6(a) shows a proposed mechanism for cleavage of a target RNA by a pRNA ribozyme chimera. The ribozyme targeting the HBV polyA signal is connected to the native 5'/3' ends of the phi29 pRNA (Fig. 6(b)). An antisense RNA, which can target intracellular DNA or RNA, is also preferred as the biologically active molecule.

$\phi$ 29 pRNA has a strong drive to form dimers (Fig. 7), and dimers are the building blocks of hexamers (Hoeprich and Guo, *J Biol Chem* 277:20794-20803 (2001); Mat-Arip et al., *J Biol Chem* 276:32575-32584 (2001); Trottier et al., *RNA* 6:1257-1266 (2000); Chen et al., *RNA* 5:805-818 (1999); Guo et al., *Mol Cell* 2:149-155 (1998); Zhang et al., *Mol Cell* 2:141-147 (1998); Hendrix, *Cell* 94:147-150 (1998)). The formation of monomers or dimers can be controlled by manipulating and controlling the sequences of the two interacting loops (Hoeprich and Guo., *J Biol Chem* 277:20794-20803 (2001); Mat-Arip et al., *J Biol Chem* 276:32575-32584 (2001); Trottier et al., *RNA* 6:1257-1266 (2000)); Chen et al., *RNA* 5:805-818 (1999); and Zhang et al., *Mol Cell* 2:141-147 (1998)).

We have shown previously that six copies of pRNA form a hexameric ring (Guo et al., *Mol. Cell.*, 2:149-155 (1998); Hendrix et al., *Cell*, 94:147-150 (1998); and Zhang et al., *Mol. Cell.*, 2:141-147 (1998)) to drive the DNA-packaging motor (see Grimes et al., *Adv. Virus Res.*, 58:255-294 (2002) and Guo, *Prog in Nucl Acid Res & Mole Biol.*, 72:415-472 (2002) for a review). pRNA dimers are the building blocks of hexamers (Chen et al., *J Biol Chem*, 275(23):17510-17516 (2000)). Hand-in-hand interaction of the right and left interlocking loops can be manipulated to produce desired stable dimers and trimers (Chen et al., *RNA*, 5:805-818 (1999); Guo et al., *Mol. Cell.*, 2:149-155

(1998); Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003); and Zhang et al., *Mol.Cell.*, 2:141-147 (1998)); hexamers are formed via hand-in-hand interaction by base-pairing of two interlocking left- and right- hand loops (Chen et al., *RNA*, 5:805-818 (1999); Guo et al., *Mol.Cell.*, 2:149-155 (1998);  
5 and Zhang et al., *Mol.Cell.*, 2:141-147 (1998)). Thus, pRNA has a strong tendency to form circular rings by hand-in-hand interaction, whether it is in dimer, trimer or hexamer form (Chen et al., *RNA*, 5:805-818 (1999) and Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003)).

The stoichiometry of pRNA has been investigated by gel,  
10 sedimentation (Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003)), binomial distribution (Trottier et al., *J.Virol.*, 71:487-494 (1997)), cryo-AFM(atomic force microscopy), and by mixing two inactive mutant pRNAs with complementary loops intermolecularly, then assaying the activity of the mixtures in DNA packaging assays (Guo et al., *Mol.Cell.*, 2:149-155 (1998)  
15 and Zhang et al., *Mol.Cell.*, 2:141-147 (1998)). The predicted secondary structure of the pRNA (Fig. 19) reveals two loops, called left- and right- hand loops (12). The sequences of the two naturally occurring loops (bases 45-48 of right hand loop and bases 85-82 of left-hand loop) are complementary. Chemical modification interference was used to distinguish the bases that are  
20 involved in intermolecular associations (i.e., dimer formation) from those which are not involved. Bases 45-49, 52, 54-55, 59-62, 65-66, 68-71, 82-85, and 88-90 showed very strong involvement in dimer formation. Chemical modification interference, chemical probing and cryo-AFM revealed that the dimer was formed via hand-in-hand and head-to-head contacts, an atypical and  
25 novel RNA dimerization that is distinct from other reported interactions such as pseudoknots or the kissing loops of HIV (Chang et al., *J Mol Biol*, 269(1):52-66 (1997); Laughrea et al., *Biochemistry*, 35(5):1589-1598 (1996); Muriaux et al., *J Biol Chem*, 271(52):33686-33692 (1996); Paillart et al., *Proc Natl Acad Sci U.S.A*, 93:5572-5577 (1996); and Puglisi et al., *Nature*, 331:283  
30 (1988)).

Chemical modification experiments suggests that  $C^{18}C^{19}A^{20}$  is present on the surface of the pRNA as a bulge used to interact with other DNA-packaging components (Trottier et al., *RNA*, 6:1257-1266 (2000)). Chemical



modification also revealed unpaired bases in loops and bulges such as bases 18-20, 42-48, 55-57, 82-86 as well as single-base bulges A<sup>9</sup>, C<sup>10</sup>, U<sup>36</sup>, A<sup>93</sup> and A<sup>100</sup>. And U<sup>72</sup>U<sup>73</sup>U<sup>74</sup> bases exist as a bulge present at the three-way junction in order to provide flexibility in folding and serve as a hinge for the twisting of the left hand stem-loop (Trottier et al., *RNA*, 6:1257-1266 (2000)). Chemical modification revealed that three of the major loops were strongly modified in monomers but were protected from modification in dimers.

To simplify the description of the subunits in the deliverable complex, we use uppercase letters to represent the right hand loop of the pRNA and lowercase to represent the left hand loop (Figs. 7, 18). The same letters in upper and lower cases indicate complementary sequences, while different letters indicate non-complementary loops. For example, pRNA A-a' represents a pRNA with complementary right loop A (5' G<sub>45</sub>G<sub>46</sub>A<sub>47</sub>C<sub>48</sub>) and left loop 'a' (3' C<sub>85</sub>C<sub>84</sub>U<sub>83</sub>G<sub>82</sub>), while pRNA A-b' represents a pRNA with unpaired right loop A and unpaired left loop 'b' (3' U<sub>85</sub>G<sub>84</sub>C<sub>83</sub>G<sub>82</sub>). See Fig. 18.

The formation of pRNA dimers (Fig. 7) might also assist in stabilizing pRNA/ribozyme chimera molecules. As long as the openings of the circularly permuted pRNAs are close to an area of dimer formation, the tertiary structure can help prevent exonucleases from accessing the ends of the RNA molecules.

When delivered systemically using prior art methods, the efficiency with which biologically active RNAs such as siRNAs and ribozymes enter the cell is very low due to the large size of the RNA. Currently, most delivery methodologies rely upon transfection and viral vectors. Chemically-mediated transfection procedures can be used in cell cultures but would clearly not be appropriate for delivery to patients. Viral vectors are efficient, but the problems in targeting to specific cells remain to be resolved.

The uptake of extracellular macromolecules and particles by receptor-mediated endocytosis (RME) is a process common to almost all eukaryotic cells. The mechanism for receptor-mediated endocytosis has been subjected to intense scrutiny and its overall feasibility for the delivery of therapeutic molecules, such as antibodies (Becerril et al., *Biochem.Biophys.Res.Comm.*, 255:386-393 (1999) and Poul et al., *J Mol.Biol.*, 301:1149-1161 (2000)), drugs

or RNA aptamers (Homann et al., *Bioorg. Med. Chem.*, 9:2571-2580 (2001)) has been reported. However, difficulties in exploiting receptor-mediated endocytosis (RME) for the targeting and delivery of therapeutic agents have been encountered and include 1) lack of specificity for the targeted cell versus  
5 healthy cells; 2) lysosomal degradation of the therapeutic molecules in the endocytic pathway; 3) instability of the targeting and delivery system in the body, and 4) adverse immunological response associated with repeated doses.

The present invention offers a mechanism for addressing the difficulties previously encountered in attempts to use receptor-mediated  
10 endocytosis for delivery of therapeutic agents. The multimeric nature of pRNA facilitates the construction of a stable, polyvalent pRNA chimera (i.e., a multimeric pRNA complex) according to the invention that carries multiple components for specific cell recognition, endosome escape, and /or delivery of one or more therapeutic molecules. A dimeric complex, for example, will  
15 contain two spacer regions and hence two biologically active moieties. For example, one of the pRNA subunits of a hexamer could carry a therapeutic ribozyme, and the other pRNA subunit could carry an RNA aptamer for surface-binding RNA (Fig. 8).

In a preferred embodiment, one subunit of the polyvalent pRNA  
20 complex carries a targeting agent, preferably an RNA aptamer (described in more detail below) or an antibody that binds cell surface receptor, thereby inducing receptor-mediated endocytosis. The targeting moiety could also interact with some component of the cell membrane or cell wall, and gain entry into the cell by a mechanism other than receptor-mediated endocytosis.

25 Another one or two subunits of the pRNA complex optionally carry components that enhance endosome disruption for the release of the delivered therapeutic molecules from the endosome. A number of substances that disrupt endosomes and mediate endosome escape of therapeutic molecules are described in the literature. Defective or psoralen-inactivated adenovirus  
30 particles have shown promise since they have considerable endosomolytic activity (Cotton et al., *Proc Natl Acad Sci U S A*, 89:6094-6098 (1992)). Synthetic peptides that mimic the membrane-fusing region of the hemagglutinin of influenza virus have also been successfully used in gene delivery systems to

facilitate endosomal escape (Mastrobattista et al., *J Biol Chem*, 277:27135-27143 (2002); Plank et al., *J Biol Chem*, 269:12918-12924 (1994); and Van Rosenberg et al., *J Biol Chem*, 277:45803-45810 (2002)). Polymeric endosome disrupting gene delivery vectors, such as poly(amino ester)(n-PAE) (Lim et al., *Bioconjug. Chem*, 13:952-957 (2002)) or poly (DL-lactide-co-glycolide) (PLGA) (Panyam et al., *FASEB J*, 16:1217-1226 (2002)) will also be tested. Endosome disrupting agents can be conveniently linked to the polyvalent pRNA complex by including one pRNA chimeric subunit that specifically bind the endosome disrupting agent. The pRNA chimera thus preferably includes, or is co-delivered with, an endosome disrupting agent such as an adenovirus. For example, the pRNA chimera can contain an RNA aptamer that binds to an adenovirus knob and thereby binds noncovalently with the adenovirus (Fig. 8).

Therapeutic agent(s) (e.g., a biologically active RNA such as a ribozyme or a siRNA, or other drug) can be carried by another of the pRNA monomers that make up a dimeric, trimeric or hexameric polyvalent pRNA chimera.

In another embodiment, an RNA or DNA therapeutic agent is encoded by the endosome-disrupting adenovirus and produced upon entry of the adenovirus into the cell. The dimeric, trimeric and hexameric polyvalent pRNA complexes of the invention are thus ideally suited for therapeutic RNAs or other chemical drugs for the treatment of cancers, viral infections and genetic diseases. Applications of multiple therapeutic agents are expected to enhance the efficiency of the *in vivo* therapy.

RNA molecules that bind other molecules (such as cell surface receptor-binding RNA molecules or RNA molecules that bind endosome disrupting agents) can, for example, be identified and isolated through SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (Tuerk et al., *Science* 249:505-510 (1990); and Ellington et al., *Nature* 346:818-822 (1990)). Such RNA molecules are known as "RNA aptamers." Starting with a library containing random RNA sequences, *in vitro* evolution techniques allow for the selection of the RNA molecules that are able to bind a specific

pre-identified substrate, such as a ligand or receptor (Ciesiolka et al., *RNA* 1:538-550 (1995); Klug and Famulok, *Molecular Biology Reports* 20:97-107 (1994). Receptor-binding ("anti-receptor") RNA can be inserted into the pRNA vector to form circularly permuted pRNA as described herein. The  
5 chimeric RNA carrying the hammerhead ribozyme and the chimeric RNA carrying the anti-receptor could be mixed to form dimers or higher order structures via inter-RNA loop/loop interaction as reported previously (Chen et al., *J Biol Chem* 275:17510-17516 (2000); Guo et al., *Mol Cell* 2:149-155 (1998); Zhang et al., *Mol Cell* 2:141-147 (1998); and Hendrix, *Cell* 94:147-  
10 150 (1998)). The use of a polyvalent RNA containing an RNA aptamer as an anti-receptor is expected to yield superior specificity compared to protein anti-receptors.

In addition, the basic principles of the SELEX method can be employed to create RNA aptamers using the basic pRNA chimera design of  
15 the invention. RNA molecules useful for the identification of RNA aptamers that bind a pre-identified substrate contain a random sequence, preferably 25 - 100 bases, present at one end of the pRNA of the present invention, preferably connected where the native 5'/3' ends are located. Optionally, linker sequences connecting the random sequence connected to both ends of the 5'/3'  
20 ends can be included. Such RNA molecules may be made by chemical or enzymatic synthesis. For instance, RNA molecules useful for the identification of RNA aptamers can be made using three primers; a template primer, a 3' end primer, and a 5' end primer (see Fig. 17). The DNA primers are designed and defined with reference to a pRNA sequence or its derivatives  
25 and counterparts. The template primer includes the random sequence flanked by two nucleotide sequences that bind the 3' and 5' end primers. Preferably, each flanking sequence of the DNA template contains a nucleotide sequence having at least 14 bases that are complimentary to the sequences of the 3' end primer and the 5' end primer corresponding to the 5' and 3' ends of the pRNA.

30 The 3' and 5' end primers can be used to make by PCR the RNA molecules useful for the identification of RNA aptamers, and also for amplification during the SELEX method. The 3' end primer contains nucleotides that are complementary to an RNA sequence to make a 5' end of a

pRNA sequence, beginning at or about at a 5' end and ending at any nascent 3'-end, e.g, base 71. Likewise, the 5' end primer contains the nucleotides that are complementary to an RNA sequence at the 3' end of a pRNA sequence, beginning at or about at a 3' end (e.g., around base 117) and ending at any nascent 5'-end, e.g, base 75 (Fig. 17). Taken together, the 5' and 3' end primers contain nucleotide sequences complementary to all or most of a pRNA sequence, preferably the wild-type pRNA sequence, such that after transcription the resultant RNA aptamer structure is that of a pRNA chimera of the invention. For example, if the 3' end primer terminates at base 71 of the wild-type pRNA, and the 5' end primer terminates at base 75 of the wild-type pRNA, only pRNA bases 72-74 will be missing from the pRNA chimera produced in the SELEX process and this will not affect the independent folding of the pRNA. The secondary structure of the resultant pRNA chimera is equivalent to the phi29 pRNA structure (see Fig. 3 for examples of equivalent structures). For example, the sequence of the 5'/3' helical region of the pRNA can vary, as long as it forms a paired double stranded region.

The RNA aptamer molecule resulting from this system, which binds the pre-identified substrate, will contain a newly selected RNA sequence connected to the original 5' and 3' end of the cp-pRNA, and will be ready for use in a variety of applications without further modification. Such RNA aptamer containing pRNA moiety will be able to bind a pre-identified substrate in variety of applications, including, but not limiting to, drug or gene delivery, and construction of nanodevices.

The SELEX system is used to identify RNA aptamers that bind specifically to proteins, polysaccharides, lipids, ATP, chemicals and theoretically any substance that has a well defined molecular structure (Bouvet, *Methods Mol.Biol*, 148:603-610 (2001); Ciesiolka et al., *RNA*, 1:538-550 (1995); Davis et al., *Methods Enzymol.*, 267:302-314 (1996); Gold, *Harvey Lect.*, 91:47-57 (1995); Kraus et al., *J Immunol.*, 160:5209-5212 (1998); Shu et al., *J.Biol.Chem.*, 278(9):7119-7125 (2003); Shultzaberger et al., *Nucleic Acids Res.*, 27:882-887 (1999); Wang et al., *Sheng Wu Hua Xue.Yu Sheng Wu Wu Li Xue.Bao.(Shanghai)*, 30:402-404 (1998); and Zhen et al., *Sheng Wu Hua Xue.Yu Sheng Wu Wu Li Xue.Bao.(Shanghai)*, 34:635-642

(2002)). Indeed, this approach can be generalized well beyond being a means to deliver an endosome disrupting agent or bind a target cell surface receptor, as it provides a way to link essentially any desired molecule (typically, a non-nucleic acid) to the pRNA delivery vehicle once an RNA aptamer that binds it has been identified. The linkage between an RNA aptamer and its target molecule is noncovalent, but cross-linking can, if desired, be achieved in some instances after the initial binding step has taken place.

Alternatively, instead of (or in addition to) using SELEX to identify RNA aptamers for specific binding, functional groups such as biotin, -SH, or -NH<sub>2</sub> can be linked to the end of the pRNA. Once the pRNA has been derivatized, endosome disrupting agents (or other desired molecules, particularly non-nucleic acid molecules) can be linked to the end of pRNA by the streptavidin-biotin interaction or by chemical crosslinking (-SH/maleimide or -NH<sub>2</sub>/NHS ester).

The ability, disclosed herein, to design pRNA molecules that assemble to hexamers in a preprogrammed, intentional manner lends unmatched versatility to the process. In addition to an anti-receptor aptamer, for example, the hexamer could harbor up to five other components. These could include poly(amino ester)(n-PAE) (Lim et al., *Bioconjug. Chem*, 13:952-957 (2002)), synthetic peptides (Mastrobattista et al., *J Biol Chem*, 277:27135-27143 (2002); Plank et al., *J Biol Chem*, 269:12918-12924 (1994); and Van Rossenberg et al., *J Biol Chem*, 277:45803-45810 (2002)), virus-derived particles (Nicklin et al., *Circulation*, 102:231-237 (2000)) for lysosome escape, adjuvants, drugs or toxins. RNA molecules are non-immunogenic. Using the same principle, dimers or trimers could be utilized. Even the hexamer-bound empty procapsid could prove useful, serving as a nanocapsule to harbor DNA coding specific genes for delivery.

The phylogenetic analysis of pRNAs from *Bacillus subtilis* phages SF5, B103, phi29, PZA, M2, NF, and GA1 shown in Fig. 3 shows very low sequence identity and few conserved bases, yet the family of pRNAs appears to have similar predicted secondary structures (Pecenкова et al., *Gene* 199:157-163 (1997); Chen et al., *RNA* 5:805-818 (1999); Bailey et al., *J Biol Chem* 265:22365-22370 (1990)). All seven pRNAs of these phages contain

both the right and left hand loops, which form a loop/loop interaction via Watson-Crick base pairing. Complementary sequences within the two loops are found in each of these pRNAs. Therefore, these pRNAs could also be used as vector to carry small therapeutic RNA molecules (Fig. 3).

5           The results from these ribozyme-mediated suppression experiments could be applied to other cell types, including those of many plant and animal species. Transgenic plants and animals could then be developed for a variety of purposes, if the chimeric ribozyme-pRNA is incorporated into the genome of cells, animals or plants.

10           Surprisingly, conjugation of a ribozyme to a bifurcated pRNA region such that both ends of the ribozyme are covalently linked to the pRNA region does not render the ribozyme inactive, nor does it appear to interfere with the independent folding of the pRNA region or the ribozyme region. Because tethering of both ends of the ribozyme RNA is expected to also prevent  
15 degradation by exonuclease, the resulting pRNA-ribozyme chimera is expected to be useful to cleave undesired RNAs in plants and animals, including humans. Additionally, transgenic plants and animals with resistance to diseases can be developed by introducing DNA encoding the pRNA-ribozyme chimera into the genomic DNA of the cell.

20           The pRNA chimera of the invention is also useful *in vitro*, for example, for the characterization of RNA molecules. RNA molecules, particularly small RNA molecules, can be stabilized or "chaperoned" by inclusion in the spacer region of a pRNA chimera of the invention, which insures that they remain properly folded, active and exposed. For example,  
25 pRNA chimera containing an RNA of interest can be immobilized, covalently or noncovalently, on a substrate, such that the RNA of interest is presented. The immobilized pRNA chimera can then be contacted with test molecules, such as cellular extracts or components, to identify the constituents to which the RNA of interest binds or otherwise interacts. This is preferable to  
30 immobilizing the RNA of interest directly on the substrate, because direct immobilization can interfere with the folding of the RNA of interest and also block portions of the structure from contact with the test molecules. The

pRNA chimera can also be used to stabilize RNAs in solution for use in binding assays, cleavage assays, diagnostics and the like.

## EXAMPLES

5

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

10

### Example 1. Elongation of phi29 pRNA at the 3' End and Effect on Activity

To investigate whether additional burdens can be imposed on the pRNA, the 3' ends of the pRNA were extended with variable lengths.

The RNA products Eco-pRNA and XbHi-pRNA were produced by *in vitro* T7 RNA polymerase transcription using DNA templates from plasmid pCRTMII that were precleaved with EcoRI or XbaI/HindIII, respectively. To generate the plasmid pCRTM2, a PCR DNA fragment was produced with the primer pair P7/P11 to flank the pRNA coding sequence (Zhang et al., *Virology* 207:442-51 (1995)). The PCR fragment was then cloned into the PCR cloning vector pCRTMII (Invitrogen, Carlsbad, CA). DNA sequencing after colony isolation confirmed the presence of the PCR fragment in the plasmid. The RNA product 174-pRNA was either extracted from procapsids, as described by Guo et al. (*Science* 236:690-94 (1987)) and Wichitwechkarn et al. (*Nucl. Acids Res.* 17:3459-68 (1989)) or transcribed *in vitro* with a PCR DNA fragment generated using the plasmid pCI3-12A(RNA) as template, following the method described in Wichitwechkarn et al. (*Mol Biol* 223:991-98 (1992)). The RNA product Di-RNA with a 120-base extension from the 3'-end of pRNA was transcribed *in vitro* with a PCR DNA fragment using cpDNAT7, as described by Zhang et al. (*Virology* 207:442-51 (1995)) as template for a PCR reaction.

It was found that at least 120 bases could be added to the 3' end of the pRNA without significant interference of pRNA function (Fig. 9). Such additions included end labeling of pRNA with biotin, pCp, DIG and



phosphate. Variable lengths of sequences added to the 3' end of pRNA had undetectable or minimal impact on viral activity. These results indicated that the 117-base pRNA folded independent of bases extended from its 3'-end.

5

#### Example 2. Circularly Permuted $\phi$ 29 pRNA

*Circularly permuted pRNA (cpRNA) from bacteriophage  $\phi$ 29 was synthesized by way of transcription from a DNA template.* The feasibility of constructing circularly permuted RNAs lies in the close proximity of the native  $\phi$ 29 RNA 5' and 3' ends (Zhang et al., *Virology* 201:77-85 (1994)).  $\phi$ 29 pRNA 5' and 3' ends are in close proximity. Construction of biologically active circularly permuted pRNAs revealed that interruption of pRNA internal bases did not affect the global folding of pRNA.

To construct circularly permuted pRNA, two tandem pRNA-coding sequences separated by a 3-base or 17-base loop sequence were cloned into a plasmid (Fig. 10) (see, Zhang et al., *Virology* 207:442-451 (1995)). Plasmids cpDNA3A (I) and cpDNAT7 (II) containing a tandem pRNA coding sequence were connected by 3- or 17-nucleotide synthetic loops, respectively. PCR was used to create dsDNA fragments with non-native 5'/3' ends. *In vitro* transcription was then performed to generate pRNAs with new 5'/3' ends. PCR primer pairs, such as P6/P5, complementary to various locations within pRNA coding sequences, were designed to synthesize PCR fragments for the transcription of cp-pRNAs. The PCR DNA fragments were directly used as templates for in vitro transcription with SP<sub>6</sub> RNA polymerase. The resulting linear cpRNA transcript linked the native 5'-end of pRNA with its 3' end by way of small loop: AAA in the case of DNA template cpDNA3A and TAATACGACTCACTATA (SEQ ID NO:8) in the case of DNA template cpDNAT<sub>7</sub>.

Fig. 10 shows generalized circularly permuted pRNA structure (SEQ ID NO:2) with arrows indicating various new openings (Zhang et al., *RNA* 3:315-323 (1997)). Wild-type sequences of 5'U1C2 and 3'A117G116 could be changed to G1G2 and C116C117, respectively, relative to wild-type pRNA to facilitate and enhance transcription by T7 RNA polymerase.

To our surprise we found that insertion of sequences to link the native 5' and 3' ends of the pRNA molecule and relocation of the 5' and 3' ends somewhere else on the molecule does not interfere with the pRNA activity, since the cpRNA was still able to catalyze  $\phi$ 29 assembly. Therefore, most of the internal bases could be used as new termini for constructing active cp-  
5 pRNA (Zhang et al., *Virology* 207:442-451 (1995); Zhang et al., *RNA* 3:315-322 (1997)).

Since linking the 3' and 5' ends of the pRNA with nucleotide sequences of variable lengths did not affect the pRNA activity, this is an indication that  
10 pRNA and the linking sequence fold independently. These findings imply that a ribozyme could be placed between the 3' and 5' ends of the pRNA could be able to fold without being influenced by the sequence and folding of pRNA.

### Example 3. *In Vitro* Activity of pRNA-Ribozyme Chimera

15 The loop used to connect the native termini of the pRNA in Example 2 did not itself possess any biological activity. However, we wondered whether an RNA sequence with biological activity would retain its activity if tethered at both ends to pRNA. It was decided to test a hammerhead ribozyme as the loop sequence.

20 An *in vitro* model system (Fig. 11) as previously described in Cotton et al. (*EMBO J.* 8:3861-3866 (1989)) was modified and used as a control to test the functionality of a pRNA-ribozyme chimera. U7snRNA (SEQ ID NO:4) was selected as the target RNA. A chimeric RNA molecule, pRNA-RzU7 (SEQ ID NO:3), was synthesized. This system was used to determine whether  
25 the pRNA could harbor other hammerhead ribozymes to function in substrate cleavage (Cotten and Birnstiel, *EMBO J* 8:3861-3866 (1989)).

30 RNAs were prepared as described previously by Zhang et al. (*Virology* 201:77-85 (1994)). Briefly, DNA oligonucleotides were synthesized with the desired sequences and used to produce double-stranded DNA by PCR. The DNA products containing the T7 promoter were cloned into plasmids or used as substrate for direct *in vitro* transcription. The anti-sense DNA encoding the U7 substrate and the DNA encoding ribozyme RzU7 were mixed with the T7 sense promoter prior to transcription. The dsDNA encoding ribozyme RzU7-

pRNA and T7 promoter were made by PCR. RNA was synthesized with T7 RNA polymerase by run-off transcription and purified from polyacrylamide gels. Sequences of the plasmids and PCR products were confirmed by DNA sequencing.

- 5           The relative abilities of the U7-targeting ribozyme (47 bases), RzU7, and the U7-targeting pRNA-ribozyme (168 bases), RzU7-pRNA, to cleave an U7snRNA fragment were compared. The ribozyme cleavage reaction was done as a control experiment to demonstrate that ribozyme reactions work correctly without any modifications. The results reveal that the RzU7-pRNA  
10   ribozyme was able to cleave the substrate with results comparable to the control RzU7 ribozyme (Fig. 12). Extended investigation revealed that specific hammerhead ribozymes harbored by pRNA, were able to cleave other respective substrates.

- The RNAs used in these experiments were generated by T7  
15   polymerase *in vitro* transcription either using PCR or by cloning into a plasmid. The transcription products are as follows:

- T7 transcription of pRNA-RzU7 yields the 168mer:  
5'GUUGAUUGGUUGUCAUAUGGCAAAAGUGCACGCUACUUUGC  
AAAACAAAUUCUAAAACUGAUGAGUCCGUGAGGACGAAAGCUGU  
20   AACACAAAAGCAAUGGUACGGUACUCCAUUGUCAUGUGUAUGU  
UGGGGAUUAACCCUGAUUGAGUUCAGCCCACAUACA3' (SEQ ID  
NO:3)

- T7 transcription of U7 template yields the 94mer:  
25   5'GGGAAAGCUUAUAGUGUUACAGCUCUUUUAGAAUUUGUCUAGC  
AGGUUUUCUGACUUCGGUCGAAAACGCCUACGUUGCAUGCCU  
GCAGGUC3' (SEQ ID NO:9)

- T7 transcription of RzU7 template yields the 47mer:  
30   5'GGCAAUUCUAAAACUGAUGAGUCCGUGAGGACGAAAGCUGUA  
ACAC3' (SEQ ID NO:10).

The abilities of RzU7 (47 bases) (SEQ ID NO:10) and pRNA-RzU7 (168 bases) (SEQ ID NO:3) to cleave U7snRNA (SEQ ID NO:9) were compared. The RzU7 cleavage reaction was done as a control experiment to demonstrate that ribozyme reactions work correctly without any  
5 modifications. The cleavage reaction using pRNA-RzU7 was done to confirm that pRNA could be successfully used as a carrier molecule for ribozymes.

The U7-targeting ribozyme RzU7 and the ribozyme RzU7-pRNA cleavage reactions were performed at 37°C for 90 minutes in the presence of 20 mM Tris pH 7.5, 20 mM MgCl<sub>2</sub>, and 150 mM NaCl. Control reactions  
10 were performed by substituting water for RNAs. The samples were dialyzed against TE (10 mM Tris, 1 mM EDTA, pH 8.0) for 30 minutes on a Millipore 0.025 µm VS type membrane. 2x loading buffer (8 M urea, TBE, 0.08% bromophenol blue, 0.08% xylene cyanol) was added to the samples prior to loading them on a 15% PAGE/8M urea denaturing gel in TBE (0.09 M Tris-  
15 borate, 0.002 M EDTA). The gel was stained with ethidium bromide and visualized using EAGLE EYE II (Stratagene).

Fig. 12(b) shows the successful results of the cleavage reaction. The predicted 69mer and 25mer cleavage products can be seen.

This experiment confirmed successfully using pRNA as a carrier  
20 molecule for ribozymes. The finding that the hammerhead ribozyme retains activity in the pRNA-RzU7 construct has important implications. Independent folding of pRNA apparently and advantageously allows the ribozyme to fold into the correct structure and perform its function in cleaving target RNA. Furthermore, since both ends of the ribozyme are connected to pRNA, the  
25 linkage is expected to protect the ribozyme from exonuclease digestion in the cell. Thus, the ribozyme will be stable after expression in the transgenic plants or animals, solving a persistent problem that has stood in the way of therapeutic use of ribozymes.

Example 4. *In Vitro* Activity of pRNA-Ribozyme Chimera against  
Hepatitis B Virus

Hepatitis is a serious disease that is prevalent in many countries  
5 worldwide. Hepatitis B virus (HBV) is one causative agent of this disease.  
HBV is an RNA virus. The RNA genome of HBV was used as target to test  
the functionality of a chimera pRNA-ribozyme. This work is important  
because it provides potential for the treatment of this serious infectious  
disease.

10 A pRNA-based vector based on bacteriophage  $\phi$ 29 was designed to  
carry hammerhead ribozymes that cleave the hepatitis B virus (HBV) polyA  
signal. This hammerhead ribozyme designed by Feng et al. (*Biol. Chem.*  
382:655-660 (2001)) cleaves a 137-nucleotide HBV-polyA substrate into two  
fragments of 70 and 67 nucleotides.

15 We tested two versions of this ribozyme: pRNA-RzA, which contained  
a pRNA moiety, and RzA, which did not. The *in vitro* plasmid pRNA-RzA  
encoding the chimera ribozyme was constructed by using restriction enzymes  
XbaI and KpnI to remove the sequence encoding the unmodified ribozyme  
from the plasmid pRzA, which encoded the ribozyme targeting the HBV  
20 polyA signal (Feng et al., *Biol Chem* 382:655-60 (2001)). Then, a dsDNA  
fragment made by PCR that encoded the 188 nucleotide chimeric ribozyme  
was ligated into plasmid pRzA that had been double-digested with Xba I and  
Kpn I (Fig. 14). The HBV-targeting ribozyme was connected to the 5' and 3'  
ends of pRNA, and the pRNA was reorganized into a circularly permuted  
25 form. Two cis-cleaving ribozymes were added to flank the pRNA and HBV-  
targeting ribozyme.

RNAs were prepared as described previously by Zhang et al. (*Virology*  
201:77-85 (1994)). Briefly, DNA oligonucleotides were synthesized with the  
desired sequences and used to produce double-stranded DNA by PCR. The  
30 DNA products containing the T7 promoter were cloned into plasmids or used  
as substrate for direct *in vitro* transcription. The *in vitro* plasmid pTZS  
encoding the HBV polyA (Feng et al., *Biol Chem* 382:655-660(2001))  
substrate was linearized with BglII. The *in vitro* plasmids encoding the HBV

polyA substrate targeting ribozyme RzA and the pRNA chimera ribozyme pRNA-RzA were linearized with EcoRI. RNA was produced by *in vitro* transcription with T7 polymerase using a linear DNA as a template for run-off transcripts. Sequences of the plasmids and PCR products were confirmed by  
5 DNA sequencing.

The product of the cis-cleaved transcript, ribozyme pRNA-RzA, was the 188mer:

5'GCUAGUUCUAGAGUUGAUUGGUUGUCAUAUGGCAAAAGUGC  
10 ACGCUACUUUGCAAAACAAAUUCUUUACUGAUGAGUCCGUGAGG  
ACGAAACGGGUCAAAAGCAAUGGUACGGUACUCCAUGUCAUG  
UGUAUGUUGGGGAUUAACCCUGAUUGAGUUCAGCCCACAUACG  
GUACCUCGACGUC3' (SEQ ID NO:17)

15 The transcribed ribozyme, RzA, is the 66mer:

5'GCUAGUUCUAGACAAAUUCUUUACUGAUGAGUCCGUGAGGACG  
AAACGGGUCGGUACCUCGACGUC3' (SEQ ID NO:18)

20 The entire cassette of the *in vitro* plasmid was under the control of a T7 promoter. During transcription of the cassette, the transcript self-cleaved to produce a chimeric ribozyme (pRNA-RzA) containing the HBV-targeting ribozyme that was connected to the pRNA (Fig. 14).

The cleavage reaction was performed at 37°C for 60 minutes in the  
25 presence of 20 mM Tris pH 7.5, and 20 mM MgCl<sub>2</sub>. pRNA-RzA (0.539 nmol) was used to cleave HBV-polyA (0.117 nmol). Control reactions were performed by substituting water for certain RNA. The RNA for which water was substituted was omitted from the name of the control. For example, the pRNA-RzA control has no HBV-polyA. The samples were dialyzed against  
30 TE (10 mM Tris, 1 mM EDTA, pH 8.0) for 30 minutes on a Millipore 0.025 µm VS type membrane. 2x loading buffer (8 M urea, TBE, 0.08% bromophenol blue, 0.08% xylene cyanol) was added to the samples prior to loading them on a 15% PAGE / 8 M urea denaturing gel in TBE (0.09 M Tris-

borate, 0.002 M EDTA). The gel was run at 100 volts until the xylene cyanol was 1.5 cm from the bottom of the gel. The gel was stained with ethidium bromide and visualized using EAGLE EYE II by Stratagene.

5 A dsDNA fragment encoding the pRNA chimera, pRNA-RzA (Table 1), was made by PCR. The pRNA-RzA ribozyme and the HBV-polyA substrate RNA were generated by *in vitro* transcription with T7 polymerase, using linear DNA as a template for run-off transcripts. This pRNA-RzA ribozyme transcription product then underwent two cis-cleavage reactions to free itself from extraneous RNA flanking sequences. "*Cis-cleavage*" means a  
10 *cleavage reaction where both the ribozyme and the substrate are part of the same molecule*. These two cis-cleavages were achieved by two ribozymes that flanked the chimera sequence. One cis-ribozyme (63 nt) was 5' to the chimera, while the other cis-ribozyme (46 nt) was 3' to the chimera (Fig. 14)). The cis-cleavage reactions predominantly occurred during the time the pRNA-  
15 RzA ribozyme was transcribed (Fig. 14).

**Table 1.** Plasmids, oligos and PCR products used for the assay of ribozyme activities

Name	Function	Promoter	Target or Purpose	Contains pRNA
cpDNA3A (plasmid)	Circularly permuted pRNA, <i>in vitro</i>	SP <sub>6</sub>	Production of cpRNA	Yes
cpDNAT <sub>7</sub> (plasmid)	Circularly permuted pRNA, <i>in vitro</i>	SP <sub>6</sub>	Construction of chimeric ribozyme	Yes
pRNA-RzA (plasmid)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	HBV polyA	Yes
pRzA (plasmid)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	HBV polyA	No
pTZS (plasmid)	Substrate, <i>in vitro</i>	T <sub>7</sub>	HBV polyA	No
pRNA-CRzA (plasmid)	Ribozyme, tissue culture	CMV	HBV polyA	Yes
pCRzA (plasmid)	Ribozyme, tissue culture	CMV	HBV polyA	No
pCdRzA (plasmid)	Disabled ribozyme, tissue culture	CMV	HBV polyA	No
p3.6II (plasmid)	HBV genomic RNAs, tissue culture		HBV polyA	No
U7 (oligos)	Substrate, <i>in vitro</i>	T <sub>7</sub>	U7	No
RzU7 (oligos)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	U7	No
PRNA-RzU7 (PCR)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	U7	Yes
12-LOX (oligos)	Substrate, <i>in vitro</i>	T <sub>7</sub>	12-LOX	No
Rz12lox (oligos)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	12-LOX	No
PRNA-Rz12lox (PCR)	Ribozyme, <i>in vitro</i>	T <sub>7</sub>	12-LOX	Yes



The processed product of the cis-cleaved transcript, a 188mer, was a major band in the gel and was purified. Examination of the gels used to purify pRNA-RzA ribozyme under UV light produced three distinct shadows. The slowly migrating band was the pRNA-RzA ribozyme. The other two bands  
5 that migrated much more quickly were the 5' and 3'-cis cleaving ribozymes. This indicates that the cis-cleavage is complete.

Cleavage of HBV-polyA substrate by the functional chimera pRNA-RzA ribozyme is shown in Fig. 13. The ribozyme pRNA-RzA, which contains a pRNA moiety, was able to cleave the substrate HBV-polyA with  
10 nearly 100% efficiency. The predicted 67 base and 70 base cleavage products are seen as one band for the cleavage reaction that included both HBV-polyA and pRNA-RzA ribozyme. The lane labeled pRNA-RzA shows a control reaction that did not contain HBV-polyA, and the lane labeled HBV-polyA shows a control reaction that did not contain pRNA-RzA ribozyme.

15 The lane labeled RzA in Fig. 13 shows two bands. The upper band (66 nt) is the ribozyme that cleaves the HBV-polyA substrate. The lower band (63 nt) is a cis-cleaving ribozyme produced in the RzA ribozyme transcription reaction. The two ribozymes migrate closely on the gels. The lane labeled RzA-pRNA shows more than one band. The top band is the chimeric  
20 ribozyme pRNA-RzA. The lower band is the cleaved products as noted above. No un-cleaved substrate was seen.

In order to use equal molar concentrations of RzA and pRNA-RzA in cleavage reaction, a large mass of pRNA-RzA was used. The other materials shown between the chimeric ribozyme and the cleaved products are degraded  
25 chimera ribozyme due to the high RNA concentration in this gel and the large size of the chimeric ribozyme. Even a small percent of degradation resulted in visible degradation products. Due to the secondary structure and incomplete denaturation by urea, the migration rate of RNAs did not match perfectly with the size.

30 It was found that the hammerhead ribozyme including its two arms for HBV targeting was able to fold correctly while escorted by the pRNA. Comparison of the cleavage efficiency of the ribozyme with and without the pRNA vector revealed a significant difference. The ribozyme pRNA-RzA,

which contains a pRNA moiety, was able to cleave the substrate HBV-polyA with nearly 100% efficiency. The chimeric ribozyme cleaved the polyA signal of HBV mRNA *in vitro* almost completely. However, the ribozyme RZA without the pRNA moiety cleaved the substrate with an efficiency much lower than 70% (not shown).

#### Example 5. Activity of pRNA-Ribozyme Chimera against Hepatitis B Virus in Cell Culture

A plasmid pCRZA was obtained from Professor Guorong Qi in Shanghai. This plasmid contains sequences coding for a cis-acting hammerhead ribozyme flanked by two sequences targeting hepatitis B virus polyA signal. When this plasmid was co-transfected into HepG2 cells with the HBV genome, HBV RNA level was decreased, and hepatitis B virus replication was inhibited in a dose dependant fashion.

We constructed a plasmid pRNA-CRZA substantially in accordance with Example 3. In pRNA-CRZA, the hammerhead ribozyme and its flanking sequence were carried by the phi29 pRNA, generating a pRNA chimera.

The design of the pRNA-CRZA plasmid used for cell culture studies was basically the same as the one used for *in vitro*, except that the CMV promoter was used instead of the T7 promoter that was used for the *in vitro* studies (Table 1). Two versions of this ribozyme were tested: pRNA-RZA ribozyme, which contained a pRNA moiety, and RZA ribozyme, which did not. Both plasmids contain sequences coding for a hammerhead ribozyme targeting the HBV-polyA signal including the two cis-cleaving hammerhead ribozymes.

The tissue culture plasmid pRNA-CRZA encoding the chimera ribozyme was constructed by using XbaI and KpnI to remove the sequence encoding the unmodified ribozyme from the plasmid pCRZA that encoded the ribozyme targeting the HBV polyA signal (Feng et al., *Biol Chem* 382:655-60 (2001)). Then, a dsDNA fragment made by PCR that encoded the 188 nt chimeric ribozyme was ligated into the position of the plasmid pCRZA that had been double-digested with XbaI and KpnI (Fig. 14).

The HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37°C and 10% CO<sub>2</sub>. Transient transfection was carried out with the method of calcium phosphate precipitation. In general, cells in 60-mm dishes were  
5 transient transfected with 1 µg of HBV expression plasmid p3.6II (Feng et al., *Biol Chem* 382:655-660 (2001)) and 5 µg of expression construct (CMV vector, pCRzA plasmid (Feng et al., *Biol Chem* 382:655-660 (2001)) or pRNA-RzA plasmid). 1 µg of pcDNA4LacZ carrying lacZ gene (Invitrogen) was also included in each transfection as internal control. β-galactosidase  
10 activity was detected to normalize the transfection efficiency among different dishes.

To analyze HBV viral RNA transcription, seventy-two hours after transfection, the cells were harvested and lysed in TRIZOL reagents (Gibco-BRL) for total RNA extraction. For northern blot, 20 µg of denatured RNA  
15 was resolved in a 0.6M formaldehyde-1% agarose gel and transferred onto HYBOND N+ nylon membrane (Amersham). Probes were prepared by random priming with the 1.8kb XbaI fragment of HBV (adr) from plasmid p3.6 II and [α-32P] dATP according to the supplier (Promega, Madison, WI). After hybridization with HBV probe, the blot was stripped and re-hybridized  
20 with a probe of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) that served as an internal control for normalizing the level of total cell RNA.

To analyze e-antigen, seventy-two hours after transfection, cells were harvested and lysed in a buffer (1% NP-40, 50mM Tris-HCl and 1mM EDTA) at 37°C for 10 minutes. Activity of β-galactosidase in cell lysate was  
25 determined to normalize the variation of transfection efficiency among different samples. The e-Ag in cell lysates and media was assayed with a commercial ELISA kit (Sino-American Co.) and normalized against β-galactosidase activity. The CMV vector, pCRzA, pRNA-RzA, and disabled ribozyme plasmid pCdRzA were transformed into HepG2 cells together with  
30 HBV expressing plasmid p3.6II and the β-galactosidase expressing plasmid pcDNA4LacZ serving as an internal control. See Table 2. The amount of CMV vector was arbitrarily taken as 1.

Table 2. Comparison of the e-antigen (e-Ag) level of HBV in medium and cytoplasm of HepG2 cells transfected with different plasmids.

5           The e-antigen assay was performed to investigate whether the pRNA could enhance the inhibition of HBV replication by hammerhead ribozyme. The e-Ag is expressed by translation from a start site upstream of the pre-core (pre-c) coding region, having a nearly identical amino acid sequence as the core antigen, while possessing different antigenicity due to the difference in  
10   location of protein expression. The e-Ag appears early during acute HBV

Plasmids	e-Ag in media		e-Ag in cell lysate		Number of experiments (n)
	$\bar{X}$ (Normalized)	S.D.	$\bar{X}$ (Normalized)	S.D.	
Vector	1	—	1	—	3
CrzA	0.790	0.072	0.816	0.176	3
pRNA-RzA	0.503	0.055	0.563	0.153	3
CdRzA	0.830	0.052	0.760	0.052	3

infection and is suitable for antigen assay in cell culture.

Assay of e-Ag revealed that pRNA enhanced the inhibition effect of ribozyme by comparing the e-Ag level of cells transfected with plasmids pcRzA (expressing hammerhead ribozyme only), pRNA-RzA (expressing the  
15   chimeric ribozyme with pRNA vector), pCdRzA (expressing the disabled ribozyme), and vector only (Table 2). The inhibition by the catalytically inactive ribozyme may be due to an antisense mechanism that involves the hybridization of arm I and arm II to the complementary HBV sequences.

To evaluate the effect of pRNA-RzA ribozyme in cell cultures,  
20   ribozyme-expressing plasmids pCRzA, pRNA-RzA, pCdRzA or empty vector was co-transfected with HBV genome-expressing plasmid p3.6 II into hepatoma HepG2 cells. The p3.6II contains 1.2 copies of HBV (*adr*) genome and produces all viral RNA transcripts (3.5Kb pre-core and pre-genomic RNA; 2.4Kb Pre-S RNA, 2.1kb S RNA and 0.8Kb X RNA) in HepG2 cells  
25   without any additional factor. Total cellular RNA was extracted seventy-two hours post-transfection. After normalizing against  $\beta$ -galactosidase activity as an internal control, comparable amounts of RNA (the amount of RNA sample

loaded in each lane can be evaluated by GAPDH level) were applied to gel and detected by Northern blotting with an HBV-specific DNA probe. The probe was used to detect the 3.5 Kb and 2.1/2.4 Kb viral RNA as indicated. The presence of pRNA-RzA ribozyme caused an obvious decrease in both 3.5  
5 and 2.1/2.4 Kb HBV RNA level.

The inhibition by this modified ribozyme was more significant compared with the CRzA ribozyme especially in affecting 2.1/2.4 Kb viral RNA level. The disabled ribozyme CdRzA (encoded by plasmid pCdRzA) bearing one base mutation in Helix II was also used in parallel with CRzA  
10 ribozyme and pRNA-RzA ribozyme (Fig. 15).

Antigen assays and Northern blot have demonstrated that phi29 pRNA can chaperone and escort the hammerhead ribozyme to function in the cell, enhancing the cleavage efficiency and inhibition effect of the ribozyme on HBV. The mechanism for such increase in ribozyme activity is probably due  
15 to the fact that the pRNA can prevent the ribozyme from misfolding and protect the ribozyme from degradation by exonucleases present in cells. The pRNA molecule contains two independently functional domains: the procapsid binding domain and the DNA-translocation domain (Fig. 2(a)). It was demonstrated that exogenous RNA can be connected to the end of the pRNA  
20 without affecting pRNA folding. At least 120 nonspecific bases were extended from the 3' end of aptRNA without hindering the folding or function of the pRNA, indicating that the 117-base pRNA was folded independent of bases extended from its 3'-end. In addition, construction of biologically active circularly permuted pRNAs revealed that interruption of pRNA internal bases  
25 did not affect the global folding of the pRNA. The demonstration that the linking of the 3' and 5' ends of pRNA with variable lengths of nucleotide sequence, which did not affect the pRNA activity, is an indication that pRNA and the linking sequence fold independently.

These cell culture studies showed that the chimeric ribozyme was able  
30 to enhance the inhibition of HBV replication when compared with the ribozyme not escorted by pRNA, as demonstrated by Northern blot and e-antigen assays. pRNA could also carry another hammerhead ribozyme to cleave other RNA substrate. These studies show that a ribozyme could be

placed between the 3' and 5' ends of the pRNA and will be able to fold without being influenced by the original pRNA sequence. These findings suggest that pRNA can be used as a vector for imparting stability to ribozymes, antisense, and other therapeutic RNA molecules in intracellular environments.

5

#### Example 6. Activity of pRNA-Ribozyme Chimera against Cancer in Cell Culture

Growth and metastasis of solid tumors requires persistent angiogenesis. Angiogenesis is a important process by which new blood  
10 vessels are formed. The protein type 12 lipoxygenase (12-LOX) in platelets makes 12-HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid) by adding O<sub>2</sub> to C-12 arachidonic acid. 12-LOX and its metabolites may be important factors in tumor angiogenesis. The application of this research could restrict tumor growth by preventing cancer cells from prompting blood vessels to grow in the  
15 surrounding tissue.

*In vitro* studies by Liu et al. have shown that this ribozyme, 12loxRz, efficiently cleaved the substrate (*Cancer Gene Ther.* 7:671-675 (2000)). Efficiency was increased when changing the reaction temperature from 37°C to 50°C. Studies in cell culture showed that cells expressing the ribozyme  
20 from a plasmid had such a decreased level of 12-LOX mRNA that it was undetectable by Northern blotting. A control group of cells that only had a nonfunctional mutant ribozyme had only a slight decrease in the level of 12-LOX mRNA. This slight reduction in 12-LOX mRNA expression could have been the result of an antisense effect by the mutant ribozyme by merely  
25 binding to the 12-LOX mRNA without cleaving it. Cell extract was assayed for 12-LOX enzyme activity. Cells expressing ribozymes had 13% of 12-LOX enzyme activity after 6 months compared to parental cells. Cells expressing the mutant nonfunctional ribozyme had 80% of 12-LOX enzyme activity compared to parental cells (Liu et al., *Cancer Gene Ther.*, 7:671-675,  
30 2000). This demonstrates the activity of the ribozyme.

Platelet-type 12-lipoxygenase (12-lox) mRNA (Fig. 16) was selected as a target to test whether a chimera hammerhead ribozyme can function to suppress mRNA levels in human erythroleukemia (HEL) cells. We obtained

the *in vitro* and tissue culture plasmids that encode the ribozyme from Professor Tien, Director of the National Key Lab of Virus Research in which the inventor Peixuan Guo is the Advisor and Visiting Professor. The hammerhead ribozyme was inserted into our pRNA essentially using the method described in Example 3. We created the chimerical ribozyme, 12loxRzpRNA, first constructing a dsDNA template in a two step PCR reaction from oligonucleotides encoding the T7 promoter and the 12loxRz inserted into the pRNA sequence. This template was subsequently transcribed to give the 12loxRzpRNA.

Experiments to test the activity of 12loxRzpRNA will be performed. For the *in vitro* experiments, the 12loxRz and a target RNA fragment of the 12-lox mRNA (the mRNA substrate) are produced from oligonucleotides essentially using the method described in Example 2. The 12loxRz and the substrate RNA are each transcribed from their own set of two hybridized DNA oligonucleotides. One encodes the negative sense T7 polymerase promoter and the substrate sequence or the 12loxRz sequence. The other oligonucleotide encodes the positive sense T7 promoter sequence. The RNA substrate is radio-labeled using calf intestine phosphatase (CIP) and then polynucleotide kinase (PNK) with [ $\gamma^{32}\text{P}$ ]-ATP.

The cleavage efficiency of two ribozymes with and without the pRNA moiety will be evaluated both *in vitro* and cells (cell culture). For the *in vitro* study, we will compare the stability of the ribozymes resistance to pH, ion concentration, RNase and cell lysate. These are factors that affect the ribozyme stability and function in the cell.

HEL cells expressing 12-lox will be used for the cell culture experiments. An empty expression cassette or the 12loxRzpRNA in an expression cassette encoding the tRNA<sup>val</sup> promoter, the 12loxRzpRNA chimera, and the eukaryote polymerase III terminator sequence (5 T residues) will be delivered by transfection using electroporation. Expression of the 12loxRzpRNA chimera and 12-lox mRNA in the cells will be detected by northern blot. Nontransfected HEL cells will be used as a control. 12-LOX enzyme activity will be evaluated by the determination of whether there is a reduction in 12-HETE production in HEL cells.

For both the *in vitro* and cell culture experiments, a mutant 12loxRz and a mutant 12loxRzpRNA chimera control will be used as a second control. The mutant 12loxRz has one of its nucleotides in its conserved catalytic core domain substituted with another base, rendering the ribozyme unable to cleave the substrate RNA. The use of the non-catalytic mutant ribozymes as a second control is designed to reveal whether the native ribozyme is capable of inhibiting translation by binding to the RNA substrate (i.e., an antisense effect), as opposed to cleaving it.

#### 10 Example 7. Construction of Active Dimers, Trimers and Hexamers

Hand-in-hand interactions between the right and left interlocking loops result in the formation of stable dimers, trimers, or hexamers. pRNA has a strong tendency to form a circular ring by hand-in-hand interaction, regardless of whether the pRNA is in its dimer, trimer or hexamer form. The sequence responsible for intermolecular pRNA/pRNA interaction is located between residues 23-97 (Chen et al., *RNA*, 5:805-818 (1999)). Change or insertion of nucleotides before residue #23 or after residue #97 does not interfere with the formation of dimers, trimers, and hexamers. The ability to form dimers or trimers is also not affected by 5' or 3' end truncation before residue #23 and after residue #97.

Our approach is to construct individual chimeric pRNA monomers that can be "mixed and matched" to carry a therapeutic agent, e.g., a "daughter" RNA molecule such as an siRNA or ribozyme, to a specific target cell. Each monomer subunit is a circularly permuted pRNA as described herein and is designed to have specific right or left loops, such as A (Right)-b'(Left), designed so as to facilitate intermolecular interactions to form a multimer. Each pRNA carries a specific "payload" (e.g., a receptor-targeting aptamer, an endosomal lysing agent, or a therapeutic RNA). Mixing of individual circularly permuted chimeric pRNA with appropriate interlocking loops results in the efficient formation of dimer, trimer or hexamer deliverable complex.



### Construction of a pRNA dimer

pRNA dimers are formed by intermolecular interaction of the interlocking right and left loops. To simplify the description of the mutants described herein, uppercase and lowercase letters are used to designate the right- and left-hand loop sequences of the pRNA, respectively. The same letter in upper and lower cases symbolizes a pair of complementary sequences. For example, in pRNA A-a', the right loop A (5'GGAC<sub>48</sub>) and the left loop a' (3'CCUG<sub>82</sub>) are complementary, while in pRNA A-b', the four bases of right loop A are not complementary to the sequence of left loop b' (3'UGCG<sub>82</sub>). Mutant pRNAs with complementary loop sequences (such as pRNA A/a') are active in phi29 DNA packaging, while mutants with non-complementary loops (such as pRNA A/b') are inactive (Fig. 20).

We found that pRNAs A-i' and I-a' were inactive in DNA packaging alone, but when A-i' and I-a' were mixed together, DNA- packaging activity was restored (Fig. 20a; Hoeprich et al., *J Biol. Chem.*, 277(23):20794-20803 (2002)). This result can be explained by the trans-complementarity of pRNA loops, i.e., the right hand loop A of pRNA A-i' could pair with the left hand loop a' of pRNA I-a'. Mixing two inactive pRNAs with interlocking loops, such as when pRNA A-b' and B-a' are mixed in a 1:1 molar ratio, results in the production of pRNA dimers with up to 100% efficiency. Thus, the stoichiometry of the pRNA is predicted to be a multiple of two (six or twelve).

We constructed several covalently linked dimeric pRNAs that were found to be active in DNA packaging in vitro (Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003)). This further verifies that dimers are the building blocks of the hexamer. Determination of the Hill coefficients of each of these three fully active RNAs implies that for each procapsid, there are three binding sites for dimers and that the binding is cooperative.

### Construction of a pRNA trimer

Another set of mutants is composed of three pRNAs: A-b', B-c' and C-a' (Fig. 20b). This set is expected geometrically to be able to form a 3-, 6-, 9-, or 12-mer ring that carries each of the three mutants. We have constructed

several sets of trimers, e.g. A-b', B-c' and C-a'. When tested alone, each individual pRNA exhibited little or no activity. When any two of the three mutants are mixed, again little or no activity was detected. However, when all three pRNAs were mixed in a 1:1:1 ratio, DNA packaging activity was restored. Indeed, stable pRNA trimers are formed with very high efficiency, up to 100%, using such sets of three interlocking pRNA (Figs. 8 and 22). The lack of activity in mixtures of only two mutant pRNAs and the restored activity in mixtures of three mutant pRNAs was expected since the mutations in each RNA were engineered in such a way that only the presence of all three RNAs will produce a closed ring. The fact that the three inactive pRNAs were fully active when mixed together suggests that the number of pRNAs in the DNA-packaging complex was a multiple of 3, in addition to being a multiple of 2 (Fig. 20b). Thus the number of pRNAs required for DNA packaging is a common multiple of 2 and 3, which is 6 (or 12, but this number has been excluded by the approach of binomial distribution and serial dilution analyses that revealed a pRNA stoichiometry of between 5-6) (Trottier et al., *J. Virol.*, 71:487-494 (1997)).

#### *Construction of a hexamer*

DNA packaging activity is also achieved by mixing six different mutant pRNAs, each of which are being inactive when used alone (Fig. 20c). Thus, an interlocking hexameric ring can be predicted to form by the base pairing of the interlocking loops. The efficiency of formation of pRNA hexamers from dimers in a protein-free solution is low (Guo et al., *Mol. Cell.*, 2:149-155 (1998) and Zhang et al., *Mol. Cell.*, 2:141-147 (1998). However, more than half of the dimer pRNA with appropriate interlocking loops could form hexamers in the presence of an appropriate protein template - the connector or the procapsid (Chen et al., *J Biol Chem*, 275(23):17510-17516 (2000) and Hoepflich et al., *J Biol. Chem.*, 277(23):20794-20803 (2002)). A hexamer with such a protein template would be useful as a delivery particle since the size of the procapsid particle is only 30nm x 40nm. The alternative approach would be to make high-yield protein-free hexamer through use of crosslinking agents incorporated into the right or left interlocking loop of the

pRNA, as reported in our previous publications (Garver et al., *J Biol Chem*, 275(4):2817-2824 (2000) and Mat-Arip et al., *J Biol Chem*, 276:32575-32584 (2001)). These hexamers are generated in the presence of protein, and the protein is removed after crosslinking in order to isolate the hexamer.

5

#### Example 8. pRNA Molecules Carrying Biologically Active RNA

Chimeric pRNA monomers can be constructed harboring desired "daughter" RNA molecules.

#### 10 *Hammerhead ribozyme*

Hammerhead ribozymes (Forster et al., *Cell*, 50:9-16 (1987) and Sarver et al., *Science*, 247:1222-1225 (1990)) target an RNA substrate sequence by using complementary nucleotides as two arms to base pair to the target RNA. Between the ribozyme's two arms of complementary nucleotides is a short sequence of catalytic RNA that performs cleaving functions against the target RNA. The target site for specific cleavage is the three-base sequence NUH (N = A, C, G, U and H = A, C, U, but not guanosine). The nucleotides on either side of the target sequence should not have a strong secondary or tertiary structure, so that the ribozyme can easily base pair to the target.

15 Methods for the selection of targets for hammerhead ribozyme action have been previously published (Mercatanti et al., *J Comput. Biol*, 9:641-653 (2002)).

A chimeric pRNA harboring a hammerhead ribozyme that successfully targeted the Hepatitis B virus RNA is described in Example 5. Transcription of the expression cassette resulted in self-cleavage of the transcript, producing a chimeric ribozyme (Example 5). To construct other chimeric pRNA harboring hammerhead ribozymes, the RNA sequence for the ribozyme are likewise connected to the 5' and 3' ends of pRNA, and the pRNA is circularly permuted, with the nascent 5'/3-end relocated preferably at residues 71/75 of the original pRNA sequence. The end at 71/75 has been shown to be located in a tightly-folded area (Hoeprich et al., *J Biol. Chem.*, 277(23):20794-20803 (2002)). The chimeric pRNA that harbors the ribozyme contains the

25  
30

appropriate right and left loops for the construction of the dimer, trimer or hexamer complex, as desired. Two cis-acting ribozymes are added to flank the pRNA and ribozyme, as reported in (Hoeprich et al., *Gene Therapy*, 10(15):1258-1267 (2003)). The entire cassette is preferably under the control of a T<sub>7</sub> promoter for *in vitro* transcription or a CMV promoter when the cassette is expressed *in vivo*.

#### *Hairpin ribozyme*

The hairpin ribozyme (Chowrira et al., *Nature*, 354:320-322 (1991) and Ojwang et al., *Proc Natl Acad Sci U S A*, 89:10802-10806 (1992)) also targets RNAs by two complementary arms base pairing to the target, but its structure and target sequence requirements are much more restrictive. The sequence requirement of a hairpin ribozyme is BN\*GUC, where B is any nucleotide other than adenine. Because a required hairpin of the ribozyme is separated from the rest of the ribozyme by one of the target binding arms, that arm is usually made to be only four nucleotides to keep the ribozyme activity reasonable. But in general, the methods and approach for the construction of chimeric pRNA monomer carrying the specific hairpin ribozyme are similar to those used for the hammerhead ribozyme.

#### *Antisense RNA*

Antisense RNAs are single-stranded RNA molecules complementary to mRNA. It has been shown that antisense RNA can inhibit gene expression in the cell (Coleman et al., *Nature*, 315:601-603 (1985) and Knecht et al., *Science*, 236:1081-1086 (1987)). We have previously demonstrated that at least 120 nonspecific bases can be extended from the 3' end of pRNA without hindering its folding and function (Hoeprich et al., *Gene Therapy*, 10(15):1258-1267 (2003) and Shu et al., *J Nanosci and Nanotech (JNN)*, 4:295-302 (2003)). Such additions included end labeling of pRNA with biotin, pCp, DIG, SH group and phosphate. Our results indicated that the 117-base pRNA folded independently of bases extended from its 3'-end. This

finding will apply to the construction of chimeric pRNA monomers carrying antisense RNA that is single-stranded. All antisense RNA used to block gene function is placed at the 3'-end of the pRNA.

## 5 siRNA

Recently, post-transcriptional gene-silencing and RNA interference have been investigated extensively in a wide variety of organisms using double-stranded RNAs (McCaffrey et al., *Nature*, 418:38-39 (2002) and Zilberman et al., *Science*, 299:716-719 (2003)). This RNA is processed into  
10 small interfering double-stranded RNAs (siRNAs) of 19-25 nucleotides (Coburn et al., *J. Virol.*, 76:9225-9231 (2003) and Elbashir et al., *Nature*, 411:494-498 (2001)), which act as guides for the formation of silencing enzymatic complex required for cleavage of the target mRNAs (Hutvagner et al., *Science*, 297:2056-2060 (2002) and Volpe et al., *Science*, 297:1833-1837  
15 (2002)). These siRNAs specifically suppress the expression of a target mRNA with a sequence identical to the siRNA. Although the detailed mechanism of post-transcriptional gene silencing and RNA interference remains to be elucidated, this powerful new technology for selective inhibition of specific gene expression employing siRNAs has shown great promise in the therapy of  
20 cancer and viral infections (Carmichael, *Nature*, 418:379-380 (2002); Li et al., *Science*, 296:1319-1321 (2002); and Varambally et al., *Nature*, 419:624-629 (2002)).

We have confirmed that the 5' and 3' ends of pRNA are paired to form a double-stranded helix (Hoeprich et al., *J Biol. Chem.*, 277(23):20794-  
25 20803 (2002) and Zhang et al., *Virology*, 201:77-85 (1994)). This double-stranded region, with more than 30 bases, is an independent domain. Complementary modification studies have revealed that altering the primary sequence does not impact pRNA structure and folding as long as the two strands are paired. We have confirmed that replacement of this double-  
30 stranded region with other double-stranded RNA does not hinder the formation of pRNA dimers, trimers and hexamers. This region could therefore be replaced by any double stranded, 19-25-base siRNA. It has been reported recently that hairpin siRNA with a loop to link both ends of the two strands of

siRNA could still function in gene silencing (Brummelkamp et al., *Science*, 296:550-553 (2002); McManus et al., *RNA*, 8:842-850 (2002); Murchie et al., *Mol. Cell*, 1:873-881 (1998); Paddison et al., *Genes Dev.*, 16:948-958 (2002); Paul et al., *Nat Biotechnol.*, 20:505-508 (2002); Sui et al., *Proc Natl Acad Sci U S A*, 99:5515-5520 (2002); and Yu et al., *Proc Natl Acad Sci U S A*, 99:6047-6052 (2002)), suggesting that it is possible to connect the siRNA at the end distal to the region for interlocking loops.

### SELEX

10        *In vitro* selection of RNA molecules that bind to specific targets has become a powerful tool for the screening of randomized RNA pools to obtain RNA molecules called "aptamers" that specifically bind to target molecules. Starting with a library containing random RNA sequences, *in vitro* evolution techniques (e.g., SELEX, Systematic Evolution of Ligands by Exponential  
15    Enrichment) allow for the selection of RNA molecules that efficiently bind to a specific receptor or ligand with high affinity (Ciesiolka et al., *RNA*, 1:538-550 (1995) and Klug et al., *Molecular Biology Reports*, 20:97-107 (1994)). Using this technique, a number of aptamers that specifically recognize many kinds of targets, such as organic compounds, nucleotides, peptides, proteins,  
20    and receptors, have been obtained.

      Though the SELEX system is powerful, one of its disadvantages is that some of the resultant RNA aptamers bind the substrate with low efficiency. Such a poor result is partially caused by the use of two primers with sequences that are pre-set values rather than being random. We developed a unique  
25    system, described herein, for using SELEX to screen RNA aptamers with stable structure and higher affinity for their targets. This system can be used to isolate RNA aptamers that bind to the cell surface receptor with both high specificity and efficiency. Such RNA aptamers are then incorporated into the pRNA via connection to the original 5'/3' end of the pRNA, and through use  
30    of an approach similar to that used for the construction of hammerhead ribozyme escorted by pRNA (Hoeprich et al., *Gene Therapy*, 10(15):1258-1267 (2003)). We have successfully constructed chimeric pRNA containing aptamers that bind to CD4 and to gp120 of HIV (Example 10). Each of these

aptamers will be selected for incorporation into a circularly permuted pRNA monomer individually.

#### Example 9. Receptor Kinase EphA2 as a Therapeutic Target

5 EphA2, a receptor tyrosine kinase, is overexpressed and functionally altered in malignant breast cancer cells. The altered EphA2 fails to bind to its ligand EphrinA1, and is responsible for the growth and invasiveness of cancer cells. It thus serves as a unique target for the specific delivery of therapeutic molecules for breast cancer therapy. RNA aptamers that bind EphA2 can be  
10 isolated by SELEX and incorporated into one of the subunits of pRNA dimer, trimer or hexamer. Indeed, receptor EphA2 has been purified, attached to a column and is being used for the screening of EphA2-binding RNA aptamer.

The pRNA multimers carrying an EphA2-specific RNA aptamer are expected to preferentially enter breast cancer cells via interaction with EphA2  
15 and endocytosis. Ribozymes or siRNA that specifically cleave mRNAs for survivin or Bcl-2, two important proteins that inhibit apoptosis of breast cancer cells, can be fused to other subunits of the pRNA multimers. Nucleotide derivatives such as 2'-F-2' deoxy CTP and 2'-F-2' deoxy UTP can be incorporated into RNA to produce stable *in vitro* RNA transcripts that are  
20 resistant to RNase digestion. These chimeric pRNAs can also be expressed by adenoviral vectors, which can specifically enter breast cancer cells via a linkage with pRNA dimers or hexamers carrying both EphA2-specific and adenoviral fiber knob-specific RNA aptamers. Chimeric pRNA (carrying the biologically active RNAs) and modified adenoviral vectors (encoding the  
25 biologically active RNAs) can be tested for their efficiency in inhibiting survivin or Bcl-2 expression in a number of breast cancer cell lines. Advantageously, adenovirus that is incorporated into the complex facilitates the release of ribozymes from the endosome.

A human adenovirus-5 recombinant expression vector that expresses  
30 the EphA2 ligand, EphrinA1, has been constructed using *Cre* recombinase-mediated Recombination System. The purified HAd-EphrinA1-Fc efficiently phosphorylates murine EphA2, leading to a drastic inhibition in EphA2 levels and also in the reduction of the tumorigenic potential of mouse breast cancer

cells. These results suggests that mouse breast cell-induced tumors in syngeneic FVB/n mice could be an excellent immunocompetent mouse model for evaluating various antitumor therapeutic approaches targeting at EphA2.

In addition, to study inhibition of the tumorigenic potential of breast cancer cells, a human breast cancer cell line (MDA-231) was infected with adenovirus expressing the extracellular domain of the ligand Ephrin-A1 attached to Fc portion of human IgG heavy chain. At various times post-infection, mock-infected or virus-infected cells were analyzed for i) levels of phosphotyrosine content; and ii) inhibition in cell viability; iii) subcellular localization of EphA2 by indirect fluorescence assay to reveal the restoration of cell-cell contacts (Fig. 21). Human Ephrin-A1-Fc expression led to EphA2 activation (tyrosine phosphorylation), which in turn led to EphA2 downregulation and significant inhibition in colony formation in soft agar. These results strongly suggest that expression of Ephrin-A1-Fc significantly inhibits the tumorigenic potential of breast cancer cells via EphA2 binding. A pRNA chimera containing ephrin as a ligand could achieve an therapeutic effect by agonizing the EphA2 receptor even without being internalized by the host cell.

#### Example 10. Construction of pRNA Dimer, Trimer, or Hexamer Complexes With Adenovirus for Specific Delivery

Adenovirus has been studied extensively as a gene delivery vector. In particular, human adenovirus type 5 (HAd5) vectors have been the focus of considerable interest in the last few years for their possible application as delivery vehicles for human gene therapy. Their therapeutic value has been hindered by the tendency of adenovirus to infect general cells rather than certain specific cell types. Recently, there has been some progress made in constructing recombinant knobs of adenovirus for the change of cell tropism (Yant et al., *Nat Biotechnol.*, 20:999-1005 (2002)), but the construction of fusion proteins to bind specific cell receptors is not as easy as making dimer pRNA that harbors a motif binding the cell receptor.



Following binding to specific cell-surface receptors, adenovirus is internalized by receptor-mediated endocytosis within a clathrin-coated vesicle (Bergelson et al., *Science*, 275:1320-1323 (1997) and Hong et al., *EMBO J*, 16:2294-2306 (1997)). The endosomal membrane fuses with adenoviral capsid, triggered by the exposure of hydrophobic residues of the adenoviral capsid proteins due to a change in endosomal pH (Seth, *Biochem Biophys Res Commun*, 205:1318-1324 (1994)). This process results in the disruption of endosomes before they are fused with lysosomes. Conjugation of naked DNA with adenovirus using transferrin or polylysine results in internalization of these conjugates by receptor-mediated endocytosis and disruption of endosomes (Cotton et al., *Methods Enzymol*, 217:618-644 (1993) and Curiel et al., *Proc Natl Acad Sci U S A*, 88:8850-8854 (1991)). It also prevents lysis of DNA by lysosomal enzymes and thus provides higher levels of foreign gene expression.

15

*Adenovirus mediated delivery of circularly permuted pRNA complexes*

We have demonstrated that DNA transfection accompanied by adenovirus infection enhances transient expression (Aggarwal et al., *Res* 63, 148-152 (1999)). The attachment of adenovirus particles to chimeric, circularly permuted pRNA thus can be employed to increase the effectiveness of release of the chimeric pRNA from endosomes, if necessary or desired. For example, an RNA aptamer can be developed which binds to an adenovirus knob, and this RNA aptamer can be incorporated into the dimers, trimers or hexamers of the complex. Expression of chimeric pRNA by adenoviral vectors is another alternative to circumvent this problem. Even mixing chimeric pRNA with replication-defective adenovirus will help in the disruption of endosomes, and accordingly help in the release of chimeric pRNA from the endosome. Inactivated adenovirus or DNA-free adenoviral procapsid particles can be incorporated into the deliverable RNA complex. The additional incorporation of the receptor-binding RNA aptamer into another subunit of the RNA hexamer is used to bring about the specific receptor-mediated endocytosis and release of therapeutic components from the endosome.

*Use of chimeric pRNA dimer to change the tropism of adenovirus for the delivery of therapeutic molecules to specific cells*

It has been shown that binding of the HAd5 fiber knob to  
5 coxsackie/adenoviral receptor (CAR) could be effectively prevented through  
use of a knob-specific antibody. For the purpose of modifying the target of  
HAd5, a knob has been linked either to a receptor-specific antibody or other  
ligands to the receptor (Bilbao et al., *Adv Exp.Med.Biol*, 451:365-374 (1998)).  
The modified virus is preferentially taken up by the specific cells. We plan to  
10 alter HAd5 tropism (or abolish the binding to receptor CAR) by constructing  
an RNA aptamer that selectively binds to the HAd5 fiber knob.

Purified HAd5 knob is used for selecting knob-specific aptamers by  
SELEX. A chimeric pRNA dimer will be constructed. One subunit of the  
dimer, trimer or hexamer will contain an RNA aptamer that binds to the cell  
15 surface receptor, such as EphA2 or gp120. The other subunit will contain an  
aptamer that binds to the human adenovirus type 5 knob. This chimeric pRNA  
dimer, trimer or hexamer can thus be used to link the adenovirus for specific  
delivery to the cell via a receptor-binding RNA aptamer. These vectors will  
fail to bind CAR (due to blocking of the binding region) but will preferentially  
20 target the specific receptor of interest (Fig. 23). Thus, HAd5 vectors will enter  
the target cells together with the therapeutic RNA complex via receptor-  
mediated endocytosis.

*Therapeutic applications of adenovirus-mediated delivery of chimeric pRNA*

25 Recombinant adenoviruses carrying ribozymes targeting at Bcl-2 to  
promote apoptosis have been examined by others (Dorai et al., *Int.J Cancer*,  
82:846-852 (1999); Perlman et al., *Cardiovasc.Res.*, 45:570-578 (2000) and  
Potter et al., *Mol.Biotechnol.*, 15:105-114 (2000)). We will combine the  
chimeric pRNA dimer with the recombinant adenovirus for the delivery of  
30 therapeutic RNA molecules to specific EphA2<sup>+</sup> cells. To determine whether  
continuous *in vivo* expression of chimeric pRNA for at least one week will  
enhance the utility of therapeutic RNA molecules, we plan to generate

replication-defective (E1 insertion) HAd5 vectors expressing chimeric pRNA containing a hammerhead ribozyme and/or siRNA targeting survivin or Bcl-2 mRNA. These vectors will be modified by linking fibers with chimeric pRNA dimers consisting of one of the pRNA subunits carrying a HAd5 knob-specific aptamer, with the other subunit having an EphA2-specific aptamer. The authenticity and kinetics of chimeric pRNA expression by HAd5 vectors in 293, MDA-231 and MCF-10A cell lines can be determined by Northern blot analysis. HAd recombinants can be purified by cesium chloride-density gradient centrifugation (Prevec et al., *J Infect Dis*, 161:27-30 (1990)) and titrated in 293 cells by plaque assay.

#### Example 11. Biotin/Streptavidin Interactions to Form Chimeric pRNA

We have developed procedures to add biotin to either the 5' or 3' end of the RNA. For 5'-end RNA labeling, we use a special promoter for T7 RNA polymerase that utilizes biotin-adenosine monophosphate as a substrate for the initiation of RNA transcription (Huang, *Nucleic Acids Res*, 31:e8 (2003)). For 3' end labeling, the pRNA complex was annealed with a synthetic biotinylated DNA oligo that is complementary to the 3' end of the pRNA. In this manner, the following exemplary particles can be incorporated into the deliverable complex: 1) fluorescent streptavidin beads with a size of 50-200 nm, incorporated into the RNA complex by biotin-streptavidin interaction; 2) phi29 procapsid (40nm) labeled with fluorescence and biotin, then linked to the RNA complex by a streptavidin molecule and purified; 3) biotinylated GFP (green fluorescent protein), linked to the RNA complex by a streptavidin molecule and then purified; and 4) streptavidin nanogold particles with a size of 5-10 nm, incorporated into the RNA complex by biotin-streptavidin interaction. In addition, the RNA can be labeled directly with fluorescence, for example 5'-labeling with Bodipy TMR-C5 (Molecular Probe) (Homann et al., *Bioorg.Med Chem*, 9:2571-2580 (2001)). The of internalization can be examined by either a fluorescence microscope or a con-focal microscope. Alternatively, the cells can be examined by flow cytometry. For the gold particle, the result is analyzed by electron microscopy.

### Example 12. Targeting HIV-infected cells

CD4 is a receptor displayed on the surface of certain T-helper lymphocytes, and is thus a unique target for the specific delivery of the deliverable RNA complex to the cell. We have constructed chimeric pRNA A-b' and B-a' monomers harboring RNA aptamers that bind CD4 (and gp120 as well). These chimeric pRNA with A-b' loops forms dimer with pRNA B-a' efficiently.

This chimeric pRNA can be incorporated into one of the subunits of pRNA dimer, trimer or hexamer. The pRNA multimers carrying a CD4-binding RNA aptamer will preferentially enter CD4<sup>+</sup> cells via interaction with CD4 and endocytosis. Ribozymes or siRNAs that specifically cleave mRNA for cellular CCR5 (Feng et al., *Virology*, 276:271-278 (2003) and Goila et al., *FEBS*, 436:233-238 (2003)), or HIV mRNAs for gag, tat (Jackson et al., *Biochem Biophys Res Commun*, 245:81-84 (2003) and Wyszko et al., *International Journal of Biological Macromolecules*, 28:373-380 (2003)), rev, env, LTR (Bramlage et al., *Nucleic Acids Res.*, 28:4059-4067 (2003)), or other locations of HIV genomic RNA are fused to other subunits of the pRNA polyvalent complex. Nucleotide derivatives can be incorporated into the pRNA to enhance the stability of RNA by conferring resistance to RNase digestion. These chimeric pRNAs can be evaluated for their efficiency in inhibiting HIV replication in a number of CD4-positive cell lines (Fig. 22). Using fluorescently labeled pRNA harboring a CD4-binding aptamer, we found that this RNA complex binds to the CD4 of a T lymphocyte.

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### Example 13. Use of a pRNA/siRNA Chimera to Silence Expression of Green Fluorescent Protein (GFP)

We made a pRNA/siRNA chimeric complex to target GFP and introduced it into a cell that expressed an exogenous GFP. We found that the chimeric pRNA complex silenced GFP expression more efficiently than the siRNA alone (Fig. 23(a)). We also used Northern blot hybridization to examine the efficiency of pRNA/siRNA chimeric complex in silencing the

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GFP target gene. We found the the chimeric complex (Fig. 23(b), lanes 1, 2, 9 and 10) silenced the GFP gene more efficiently than the siRNA alone (without the pRNA delivery vehicle) (Fig. 23(b), lanes 3 and 7). Lanes 4 and 8 are controls and show the GFP mRNA in the absence of treatment with siRNA.

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#### Example 14. Use of a pRNA/Ribozyme Chimera to Target Survivin

We made a pRNA/ribozyme chimeric pRNA complex to target survivin and introduced it into a cancer cell. Survivin is a gene product that plays a role in opposing programmed cell death (apoptosis). We found that the chimeric pRNA complex led to apoptosis, causing cancer cells to die (Fig. 24)

#### Sequence Listing Free Text

	1	organism name: Bacteriophage phi29/PZA
15	2	circularly permuted pRNA from bacteriophage phi29 (short loop)
	3	RNA chimera containing phi29 pRNA and hammerhead ribozyme
	4	U7snRNA substrate
	5	anti-12-Lox ribozyme
	6	Lox substrate RNA
20	7	pRNA chimera
	8	linking loop
	9	U7 substrate
	10	RzU7 hammerhead ribozyme
	11	organism name: Bacteriophage SF5'
25	12	organism name: Bacteriophage B103
	13	circularly permuted pRNA from bacteriophage phi29 (long loop)
	14	organism name: Bacteriophage M2/NF
	15	organism name: Bacteriophage GA1
	16	aptRNA
30	17	RNA chimera containing phi29 pRNA and hammerhead ribozyme
	18	RzA hammerhead ribozyme
	19-22	3' pRNA extension

- 23 hammerhead ribozyme
- 24 Hepatitis B virus polyA substrate
- 25 RNA chimera containing phi29 pRNA and hammerhead ribozyme
- 26 Wild-type pRNA with base pair change at base of stem structure.
- 5 27 Wild-type pRNA

The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described; many variations will be apparent to one skilled in the art and are intended to be included within the invention defined by the claims.